Synbiotic efficacy of probiotic and prebiotic food ingredients for gut health

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STATEMENTS AND DECLARATIONS

Statement of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. All animal experiments conducted in this thesis were done under the approval of the University of Tasmania's Animal Ethics Committee; animal ethics approval number **A0015840**.

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PUBLICATIONS AND STATEMENT OF CO-AUTHORSHIP

This thesis includes work, which has been published or to be submitted for publication in a peer-review journal. More details for each paper are described below. The following people and institutions contributed to the publication of work undertaken as part of this thesis:

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Communications arising from this thesis

Paper 1: **Probiotic *Bacillus coagulans* MTCC 5856 spores exhibit excellent *in-vitro* functional efficacy in simulated gastric survival, mucosal adhesion and immunomodulation.**

Authors: **Tanvi Shinde**, Ravichandra Vemuri, Madhur D Shastri, Agampodi Promoda Perera, Stephen Tristram, Roger Stanley, Rajaraman D Eri.

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Candidate was the primary author and contributed to the conception and design of the research project, conducted all the experiments, analysed the results and wrote the manuscript (80%). Author 1 (5%), Author 2 (5%) and Author 3 (5%) contributed to the conception and design and critically reviewed the manuscript. Author 4 and 5 (5%) assisted in the experiments.

Paper 2: **Synbiotic Supplementation Containing Whole Plant Sugar Cane Fibre and Probiotic Spores Potentiates Protective Synergistic Effects in Mouse Model of IBD.**

Authors: Tanvi Shinde, Agampodi Promoda Perera, Ravichandra Vemuri, Shakuntla V. Gondalia, Avinash V. Karpe, David J. Beale, Sonia Shastri, Benjamin Southam, Rajaraman Eri and Roger Stanley

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Parts of this thesis have contributed to publications of which the candidate is the primary author. Listed above are these publications, along with author contributions. In all cases the material included in the thesis was performed by the candidate, except where due acknowledgement is made.

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Conference Presentations

Oral presentations:

Shinde T., Stanley R. and Eri, R. Probiotic and prebiotic combinations for gut health. *Tasmanian Health Research Conference*, Hobart, Australia. 4th July 2016.

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Shinde T. Improving gut defence for soldiers. *Asia-Pacific Three-Minute Thesis*, University of Queensland, Brisbane, Australia. 30th September 2016.

Shinde T., Tristram S., Stanley R. and Eri R. Survival, adhesion and immunomodulatory efficacy of spore-forming probiotic *Bacillus coagulans*. *Australian Society for Microbiology National Scientific Meeting*, Hobart, Australia. July 2- 5, 2017.

Shinde T., Eri R. and Stanley R. Efficacy of synbiotic combination containing probiotic spores and prebiotic sugar cane flour in experimental colitis. *12th International Scientific Conference on Probiotics, Prebiotics, Gut Microbiota and Health – IPC2018*, Budapest, Hungary. June 19-21, 2018.

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Shinde T., Stanley R. and Eri R. Defence synbiotic snack bar for improved gut health. *Tasmanian Health Research Conference*, Hobart, Australia. 4th July 2016. (1St Prize).

Shinde T., Eri, R. and Stanley R. Probiotic, prebiotic and synbiotic for gut health. *Connect North-Research Expo, Spotlight Symposium : Research in North*, University of Tasmania, Launceston, Tasmania, Australia. 29th August 2016.

Patents

The information from this study has been filed as a provisional patent application in Australia titled "Preparation for the Treatment of Inflammatory Bowel Disease using a Whole Plant Fibre Extract from Sugar Cane" with application number 2018902145 and a filing date of 15 June 2018. Information relating to novelty of synergy between probiotic *Bacillus coagulans* and prebiotic whole plant sugar cane fibre in imparting health benefits is the subject of the patented claim.

Prepared manuscripts for publication

Chapter 5: Prebiotic green banana resistant starch and probiotic *Bacillus coagulans* spores in synbiotic supplementation ameliorates gut inflammation in mouse model of IBD

Chapter 6: Efficacy of sugar cane fibre and probiotic spore synbiotic combination in attenuating colonic inflammation in Winnie mice

Publications related to but not directly arising from this thesis

Vemuri R., Gundamaraju R., **Shinde T.**, Perera A.P., Basheer W., Southam B., Gondalia S., Karpe A., Beale D., Tristram S., Ahuja K., Ball M., Martoni C., Eri R. *Lactobacillus acidophilus* DDS-1 Modulates Intestinal-Specific Microbiota, Short-Chain Fatty Acid and Immunological Profiles in Aging Mice. Nutrients, 2019. **11**(6): 1297.

Perera A.P., Fernando R., **Shinde T.**, Gundamaraju R., Southam B., Sohal S. S. Roberstson A., Schroder K., Kunde D., and Eri R. MCC950, a specific small molecule inhibitor of NLRP3 inflammasome attenuates colonic inflammation in spontaneous colitis mice. *Scientific reports,2018*. *8*(1): 8618.

Vemuri R., **Shinde T.**, Gundamaraju R., Gondalia S., Karpe A., Beale D., Martoni C., Eri R. *Lactobacillus acidophilus* dds-1 modulates the gut microbiota and improves metabolic profiles in aging mice. *Nutrients*, 2018. 10(9): 1255.

Vemuri R., **Shinde T.**, Shastri M., Perera A.P., Tristram S., Martoni C., Gundamaraju R., Ahuja K., Ball M., Eri R. A human origin strain Lactobacillus acidophilus DDS-1 exhibits superior in vitro probiotic efficacy in comparison to plant or dairy origin probiotics. *International Journal of Medical Sciences*, 2018. 15(9): 840-848.

Vemuri R., Gundamaraju R., **Shinde T.** and Eri R. Therapeutic interventions for gut dysbiosis and related disorders in the elderly: antibiotics, probiotics or faecal microbiota transplantation? *Beneficial microbes*, 2017. 8(2): 179-192.

DEDICATION

I wish to dedicate this thesis to the most loving people in my life – my husband Sandesh, daughter Shanaya and my parents Meena and Govind for their endless love, support and encouragement. Thank you all for giving me the strength to reach for the stars and chase my dreams. My parents not only raised and nurtured me in the best possible ways but have always encouraged and supported my interests throughout. Completing this degree meant a huge amount to me since my mother never had the opportunities that I have been given. Thanks Sandesh and Shanaya for being the immense sources of motivation and strength during moments of despair and difficulties. Your love and care have been shown in incredible ways. Thank you for believing in me more than I do.

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This thesis is only a beginning of my journey.

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ABBREVIATIONS

ANOVA	**Analysis of variance**
APCs	**Antigen presenting cells**
CD	**Crohn's disease**
CFU	**Colony forming units**
CRP	**C-reactive protein**
DAI	**Disease activity index**
DC	**Distal colon**
DCs	**Dendritic cells**
DF	**Dietary fibre**
DSS	**Dextran sulfate sodium**
FOS	**Fructo-oligosaccharides**
GBF	**Germinated barley foodstuff**
GBRS	**Green banana resistant starch**
GC-MS	**Gas chromatography-Mass spectrometry**
GIT	**Gastrointestinal tract**
GPRs	**G-protein-coupled receptors**
GRAS	**Generally regarded as safe**
HC	**Healthy control**
H&E	**Haematoxylin and eosin**
IBD	**Inflammatory bowel diseases**
IBS	**Irritable bowel syndrome**
IFN	**Interferon**
Ig	**Immunoglobulin**
IL	**Interleukin**
InChI	**International chemical Identifiers**
iNOS	**Inducible nitric oxide-synthase**
KEGG	**Kyoto encyclopaedia of genes and genomes**
LAB	**Lactic acid bacteria**
LDA	**Linear discriminant analysis**
LEfSe	**Linear discriminant effect**
LPS	**Lipopolysaccharides**
MIP	**Macrophage inflammatory protein**
MPO	**Myeloperoxidase**
Muc 2	**Mucin 2 gene**
NFκB	**Nuclear factor-κB**
NK	**Natural killer**
OPLS-DA	**Orthogonal partial-squares discriminant analysis**
OTU	**Operational taxonomic units**
PB	**Probiotic bacteria**
PC	**Proximal colon**
PCoA	**Principal coordinates analysis**
PCW	**Plant cell walls**
PSCF	**Prebiotic sugar cane fibre**
RS	**Resistant starch**
SAM	**Significance analysis for microarrays**
SCFA	**Short chain fatty acids**
SGJ	**Simulated gastric juice**
SIJ	**Simulated intestinal juice**
SSJ	**Simulated salivary juice**
TGF	**Transforming growth factor**
Th	**T-helper cells**
TJ	**Tight junction**
TNBS	**2,4,6-trinitrobenzene sulfonic acid**
TNF	**Tumour necrosis factor**
TLR	**Toll-like receptors**
Treg	**T- regulatory cells**
UC	**Ulcerative colitis**
VIP	**Variable importance of projection**
WT	**Wild-type**
ZO-1	**Zonula occludens-1**

ABSTRACT

The main objective of this study was to test the efficacy of synbiotic food combinations carrying probiotic and prebiotic dietary fibres (DF) components for mitigating colonic inflammation that is associated with gut health issues such as inflammatory bowel disease (IBD). Although the exact aetiology of IBD is yet to be elucidated, emerging evidence supports the involvement of a recurrent tripartite pathophysiological circuit encompassing dysregulated immune responses, altered epithelial integrity and microbial dysbiosis. Therefore, the potential of dietary interventions incorporating food combination synergism to mitigate the inflammatory circuit, and thereby resolve or prevent the severity of colonic inflammation, was investigated. Whole plant prebiotic sugar cane fibre (PSCF) and green banana resistant starch (GBRS) flour prebiotics were evaluated for their individual as well as synbiotic efficacy in combination with probiotic *Bacillus coagulans* MTCC 5856 spores (*B. coagulans*) for ameliorating chemically-induced acute colitis and spontaneous chronic colitis in mice models of IBD.

The research initially determined the stability and the bioefficacy of *B. coagulans* spores *in-vitro* by evaluating their ability to survive simulated digestion, adhesion to human colonic epithelial cells and immunomodulatory capacity. The tolerance of the probiotic *B. coagulans* spores to simulated digestion was tested by exposure to simulated saliva, gastric and intestinal juices. There was a high survival rate of 92% to the simulated digestion process. There was also substantial adherence to human colonic cells HT-29 (86%) and LS174T (81%). Furthermore, the spores exerted marked immunomodulatory effects in HT-29 cells by suppressing IL-8 and increasing IL-10 secretion. The *B. coagulans* spores also induced a pronounced differential immunomodulatory efficacy in response to lipopolysaccharide-induced inflammation under co-treatment (increased IL-10 and reduced IL-8) relative to post-treatment (suppressed IL-8 with no IL-10 detection) in HT-29 cells. These observations support the application of *B. coagulans* spores prior to or during the onset of inflammation to maximise the probiotic benefits in treating inflammatory bowel conditions

The prophylactic efficacy of dietary supplementation with *B. coagulans* spores and PSCF alone or as synbiotic combination was then evaluated for their ability to attenuate dextran-sulfate sodium (DSS)-induced acute colitis in C57BL/6J mice. The study also aimed to analyse the beneficial effects of pre-conditioning the gut with supplemented diets prior to

induction of chemical colitis in imparting protection and amelioration against DSS-induced acute colitis. The mice were fed a normal chow diet supplemented with either whole plant PSCF alone, *B. coagulans* or its synbiotic combination (PSCF-synbiotic). The mice in control group (DSS-control) received normal chow. After the first seven days of supplementation, acute colitis was induced with 2% DSS administered in drinking water for seven days with the continuation of the supplementations. The disease activity indices (DAI), macroscopic markers, histological colonic damage, expressions of tight junction (TJ) proteins, mucus staining were analysed. The profile of colonic and serum cytokines and other inflammatory mediators (colonic iNOS activity and serum C-reactive protein level) were determined. Additionally, the faecal metabolomic and short-chain fatty acids (SCFA) (caecal, mucosal-associated and faecal samples) profiles were also measured. Synbiotic supplementation ameliorated DAI and histological score (72% reduction, 7.38, respectively), more effectively than either *B. coagulans* (47% reduction, 10.1) and PSCF (53% reduction, 13.0) alone relative to the DSS-control. PSCF-synbiotic supplementation also significantly ($P < 0.0001$) preserved the expression of TJ proteins and modulated the altered serum IL-1β (−40%), IL-10 (+26%), and C-Reactive protein (CRP) (−39%) levels compared to the unsupplemented DSS-control. DSS insult in control mice resulted in decreased expression of TJ proteins and altered immune responses. Moreover, *B. coagulans* spores alone induced extra butyrate production in the caecum (+81%), but only +17% in mucosal-associated samples and +44% in faeces relative to DSS-control group. In contrast, the synbiotic combination resulted in substantial increase in butyrate levels across the whole length of colon with +80% in caecum, +57% in mucosal-associated sample and +54% in faeces.

The ability of *B. coagulans* spores and GBRS alone and as synbiotic combination (GBRS-synbiotic) was then examined for prophylactic efficacy in influencing the onset and disease outcomes of DSS-induced acute colitis in C57BL/6J mice. This study employed the same design where, after the first seven days of supplementation, acute colitis was induced with 2% DSS administered in drinking water for seven days with the continuation of the supplementations. The GBRS-synbiotic supplementation alleviated the DAI and histological damage score (67% reduction, 8.8 respectively) more than *B. coagulans* (52% reduction, 10.8 respectively) or GBRS (57% reduction, 13.6 respectively) alone. Compared to the DSS-control, synbiotic supplementation significantly ($P < 0.0001$) maintained the expression of TJ proteins. Moreover, synbiotic effects accounted for approximately 40% suppression of IL-1β and 29% of the increase in serum IL-10 while also reducing CRP (37%) to that of the DSS-control. Additionally, relative to that of DSS-control, GBRS-synbiotic supplementation also

significantly raised the SCFA profile especially faecal butyrate level (+66%) more extensively compared to *B. coagulans* supplementation alone (+46%). Both the studies demonstrated marked prophylactic efficacy of the synbiotic supplementations (PSCF-synbiotic and GBRS-synbiotic) in ameliorating the acute colitis in mice.

A further study employed a spontaneous colitic Winnie (Muc2 mutant) mice model of IBD to determine if the previous results were specific to the DSS model or more generally applicable. In the Winnie model the chronic colonic inflammation results from a primary intestinal epithelial defect conferred by a missense mutation in Muc2 mucin gene. Winnie mice were fed normal chow diet supplemented with either *B. coagulans*, PSCF or its synbiotic combination for 21 days. Prominent features of the spontaneous colitis in Winnie, such as severe clinical manifestations, colonic histological alterations, dysregulated immune responses, altered SCFA levels and microbial dysbiosis, were examined to determine the therapeutic effect of the supplementations in mitigating chronic inflammation. All three supplementations reduced diarrheic stools as well as prevented body weight loss. PSCF-synbiotic supplementation significantly ameliorated histological score in both proximal ($P = 0.0443$) and distal ($P < 0.0001$) colon sections more effectively than *B. coagulans* and PSCF alone. Moreover, PSCF-synbiotic supplementation substantially modulated the altered colonic and serum cytokine levels as well as lowered serum CRP level by 29% compared to the unsupplemented Winnie-control. While, PSCF favoured the abundance of the bacterial genus *Akkermansia*, PSCF-synbiotic was effective in restabilising the depleted levels of *Prevotella* in Winnie colitic mice. PSCF-synbiotic was also markedly effective in elevating and normalising the levels of short-chain fatty acids along the length of the colon compared to that in unsupplemented Winnie-control mice.

The potentiated health outcome effects of the synbiotic combinations observed in these studies may be associated with a synergistic direct immune-regulating efficacy of the probiotic and prebiotic components. The synbiotic combinations may also exert their effects by improved ability to protect epithelial integrity, stimulation of probiotic spores by the respective prebiotic fibre and/or with stimulation of higher levels of fermentation of fibres releasing SCFAs that mediate the reduction in colonic inflammation. Thus, the synergism between the probiotic and prebiotic components used in these studies could be attributed to the observed augmented beneficial effects. The knowledge obtained from thesis not only warrants investigation of synbiotic supplementations as an adjuvant therapy in human IBD,

but the results could also be applied to design novel functional food products targeted at improving gut health and enhanced eating practice.

Chapter 1
Introduction

1.1 Significance of the topic

Food is being increasingly appreciated as an important vehicle carrying health-promoting bioactives to reduce the risk of diseases (1) and is no longer merely a source of essential nutrients to satisfy human hunger. The choice of healthy foods and improved eating practices can facilitate a paradigm shift from illness to wellness. With the gradual change in consumers' perspective on food there is a huge demand for foods possessing health benefits beyond basic nutrition. This trend directs research towards the development of functional foods carrying probiotic bacteria (PB) and prebiotic dietary fibres (DF) for optimal wellness (2, 3). Considering the preference for "convenience food" among consumers (4), the challenge however, is to deliver these functional food ingredients in convenient ready-to-eat formats whilst preserving their optimum bioactivity. Shelf stable food formats fortified with probiotic and prebiotic ingredients are also required for application in defence and space programs or similar where long shelf life at ambient is needed (5-7). Additionally, limited availability of fresh, nutrient-dense food during deployment and space voyages also creates challenges to gut health resilience. The occurrence of gastrointestinal disorders can be promoted due to weakened immunity resulting from the environmental stresses (7, 9, 10). Functional food formats that can withstand wide temperature ranges, have long shelf life and are light weight are considered ideal for inclusion in meal packages for troops (8). Hence, delivering effective doses of probiotic and prebiotic ingredients through suitable food matrices is needed. Careful selection of compatible bioactive ingredients and understanding the mechanisms of bioefficacy will facilitate the development and optimisation of these functional probiotic and prebiotic shelf-stable foods.

This research aimed at developing symbiotic combinations of functional food ingredients to lead the development of shelf stable value-added functional foods with anti-inflammatory potential. The study focussed on understanding the interactions and mechanisms of actions of synergistic synbiotic combinations of probiotic spores and whole-plant prebiotic DF in influencing the gastrointestinal health using animal models. Furthermore, the shelf-stable nature of such synbiotic food formats would allow storage at extended periods without refrigeration, suitable for combat ration packs and spaceflight meal packs. Additionally, improvement in the nutritional and functional value of such shelf stable

foods by fortification with anti-inflammatory food ingredients are likely to impact the gut health of civilians through enhanced eating practices.

1.1.1 Problem background and the purpose of the study

Gastrointestinal health is a strong determinate of an individual's overall wellbeing and studies have demonstrated the significance of diet in influencing gut health (9-11). Diet is also a major influence on the composition of gut microbiota (12-14). As the role of gut microbiota is being increasingly understood and highlighted in health and diseases (12, 13, 15, 16) tools to manipulate the gut microbial profile are being sought. This has opened new research avenues to modulate health using functional foods as a vehicle for carrying health-promoting bioactives to prevent the risk or treat diseases (17) including gastrointestinal pathologies. Chronic inflammation of the gut is a hallmark feature of lifestyle diseases including inflammatory bowel diseases (IBD), obesity and related comorbidities (18). Such inflammatory conditions encompass overlapping features including altered chronic inflammation, weakened barrier integrity and microbial disturbances (dysbiosis). This study aimed to demonstrate the anti-inflammatory potentials of functional food ingredients employing animal models of IBD as a prototype of gut inflammation.

Inflammatory bowel diseases (IBD) are a group of chronic relapsing gastrointestinal tract (GIT) disorders including ulcerative colitis (UC) and Crohn's disease (CD) that are characterized by inflammation of the GIT and imbalance or dysbiosis of the gut microbiota (19). The incidence of CD and UC is rising globally (9, 19). Despite current medical treatments that focus primarily on immunosuppression (20), overall 20% of patients with CD still require surgery and over 10% of UC patients still require colectomy (21). This highlights the urgent need for research into prevention and management of these complex pathologies to avoid debilitating complications and the need for substantial risk medical interventions.

The adoption of "Westernised" diet low in fruits and vegetables has been blamed as a potential factor for the recent rise in IBD incidence (9, 22). Even though the aetiology of IBD still remains unclear, involvement of three distinct recurrent features have been clearly identified that includes, gut dysbiosis, dysregulated immune response and altered colonic epithelial integrity (19). Application of preventive or therapeutic approaches that target this recurrent inflammatory cycle are needed. Dietary interventions are increasingly perceived as both preventive and corrective strategies to break the inflammatory cycle owing to their

ability to interact with the immune system, and to influence the gut microbiota composition and associated bacterial metabolic pathways (11, 23-27). In this context, dietary components such as PB and prebiotic dietary fibre (DF) are thought to be useful leading to potential resolution or prevention of IBD. The potential of these bioactive ingredients in ameliorating inflammation in the gut may be associated with their abilities to modify gut microbiota composition and metabolites, regulate secretion of immunomodulatory molecules and strengthen the colonic epithelial integrity (19, 26, 28).

Probiotics are defined as "live micro-organisms which, when, administered in adequate amounts, confer a health benefit on the host" (29). Therapeutic efficacy of PB in treatment of GIT conditions including diarrhoea, irritable bowel syndrome and IBD have been demonstrated (30). The benefits from probiotic therapy however, is largely governed by the species and the strains used (31). It is clear that not all probiotics are equally beneficial since each may have an individual mechanism of action and differences in host characteristics may determine which probiotic species and strains could be effective (32-35).

Maintenance of viability and functionality of PB during the industrial processing and storage, in the food/pharmaceutical preparations and during gut transit is thought to be necessary (36, 37). However, certain strains of commonly applied probiotics such as *Lactobacillus* and *Bifidobacterium* are reported to be sensitive to gastric transit and do not survive during the shelf-life of the products (38-41). From this perspective, probiotic *Bacillus* species that form spores offer advantage over other conventionally used PB in terms of survival in harsh conditions during processing, storage and gastric transit (42-45). A number of reports have demonstrated the probiotic efficacy of *Bacillus* spores in exerting immunomodulation and pathogen exclusion attributes in animals and humans (46, 47). Moreover, certain probiotic *Bacillus* species have been confirmed to confer anti-diarrheal effect (48, 49). These characteristics warrant research on their potential application in IBD to mitigate clinical manifestations and inflammation and to understand their mechanisms of action.

For probiotics to be beneficial and confer sustained positive effects, they need to be present from either continued ingestion or from having an effective prolonged residence time in the gut. Therefore, promoting the survival and activity of the ingested probiotics to ensure greater number reach the colon and/or enhance their residence time and activity in the colon is

a viable strategy (50). This can be facilitated by prebiotics, which when combined with probiotics, can form an advantageous combination known as synbiotics (24).

Prebiotics typically refer to selectively fermented non-digestible food ingredient, fibre or substances that pass the small intestine undigested and specifically support the growth and/or activity of health-promoting bacteria that colonize the colon (24, 51). Prebiotic dietary fibres (DF) have shown particular promise in attenuating colonic inflammation in humans (11, 23). While the underlying mechanisms of DF are thought to be multifactorial, major potential mechanistic contributors to its beneficial effects are the dilution of toxins via stool bulking and the production of metabolites through bacterial fermentation, particularly short chain fatty acids (SCFA) (23).

The health efficacy of DF may vary by the type of DF. Most studies examining the efficacy of DF have focused on purified ingredients that represent limited complexity in contrast to those that naturally occur in fruits and vegetables (52). However, enough evidence exists, and is recently being more recognized, to indicate that the actual biochemical complexity of naturally occurring DF such that in fruits and vegetables, is an important attribute in governing the microbial complexity of GIT (52-54). This highlights the prudence of developing functional food applications where the prebiotic fibres are representative of those in whole plant vegetables and fruits, and thus retain fibre biochemical complexity.

In addition to modulating the gut microbiota, the prebiotic ingredients improve the survival, activity and/or metabolism of the target PB in the colon by selectively stimulating its growth when applied as synbiotic (55). Synbiotics which are combinations of probiotic and prebiotic ingredients, in functional foods could potentially confer augmented prophylactic and therapeutic effects to the host owing to either complementary and/or synergistic outcomes. Such two-point approach could possibly be more successful in delivering the required health benefits by impeding more than one inflammatory circuit components of IBD. Moreover, prebiotics have been confirmed to improve product stability during storage by influencing the survival of PB in the product during the shelf life (56). Thus, functional synbiotic foods that encompasses the synergy between the probiotic and prebiotic ingredients will be pragmatic strategy to achieve potentiated gut-health promoting benefits and influence enhance eating practices.

1.1.2 Research value of the study

This study has evaluated the efficacy of DF and probiotic combinations in ameliorating acute and chronic colitis in murine models of IBD. The study employed probiotic *Bacillus coagulans* MTCC 5856 (*B. coagulans*) spores and two different prebiotic dietary fibres: whole plant prebiotic sugar cane fibre (PSCF) and green banana resistant starch (GBRS) flour. *In-vitro* cell culture screening was initially used to assess probiotic potential of the *B. coagulans* spores through measuring survival during simulated digestion, attachment to human colonic epithelial cells and immunomodulatory capacity. An IBD model using chemically induced colitic mice treated with *B. coagulans* spores alone or as synergistic synbiotic combination with either PSCF or GBRS was then used to determine their beneficial protective effect in mitigating acute inflammation. A further IBD model using chronic colonic inflammation in spontaneous colitic mice was also used to evaluate synergistic synbiotic treatment containing *B. coagulans* and PSCF.

The delineation of the mechanism of action of the synergy between the synbiotic components is important to allow development of effective synergistic shelf-stable functional food design. While the research in this study was targeted to functional food supplements it is recognised that the results could also be applied to design novel commercial fresh food synbiotic products targeted at improving human gut health and enhanced eating practices. However, the scope of the research was limited to shelf stable formulations. The wider functional ingredient options of other non-shelf stable probiotics and other more natural and less concentrated sources of prebiotics was excluded although they may allow other health benefits and delivery forms to be effective in promoting gastrointestinal health.

1.2 Research Strategy

This thesis aims to determine the efficacy of probiotic and prebiotic combination (synbiotic) for improving gut health. The rationale behind the choice of *Bacillus coagulans* spores as probiotic, whole plant prebiotic sugar cane fibre (PSCF) and green banana resistant starch (GBRS) flour as prebiotic related to the goal of developing shelf stable functional food supplements. The desire to study the efficacy of prebiotic and probiotic in synbiotic combination stemmed from the need to understand synergistic functioning of these bioactive components in order to efficiently achieve augmented beneficial health outcomes with condensed forms of shelf-stable functional foods. While, numerous studies have confirmed health benefits from the individual application of probiotics and prebiotics (57-61), only some

reports support the application of synbiotic combinations for achieving added health benefits (26, 28, 55, 62). Additionally, studies delineating the mechanism of synergistic interaction and bioefficacy of probiotic and prebiotic components are scarce. Elucidation of the synergistic mechanism is vital to the understanding of the factors and attributes involved in achieving the augmented benefits and optimisation of the beneficial synergism. The information on the mechanisms of the synergy will then allow further development or enhancement of therapies or food products for targeted health benefits as well as possible applications into improved eating practices. However, a careful selection of the compatible probiotic and prebiotic components was required to achieve the potentiated synbiotic benefits. In addition, selection of biochemically complex DF and stable probiotic that survives the gastric transit, manufacturing and storage temperatures was deemed important to ensure effective dose reaches the colon.

The outcome goal of this study was to develop food products that would be shelf stable without refrigeration and retain efficacy over a prolonged shelf life. To achieve this, the study explored the application of probiotic spores considering the benefits of spores for retaining viability and functionality of PB during the industrial processing, storage and shelf-life of the product. The ability of *Bacillus* species to form spores confers this higher resistance to technological stresses encountered during industrial production and storage processes as well as a greater protection against the hostile gastric and intestinal conditions (63-66). *Bacillus coagulans* also has known health-promoting, anti-diarrhoeal and safety attributes (48, 67-69). Additionally, for legislative compliance, the *Bacillus coagulans* MTCC 5856 spores used in the study are GRAS (Generally Recognised as Safe) probiotics with Food Standards Australia New Zealand (FSANZ) specifically permitting *B. coagulans* for probiotic application in food (70). *B. coagulans* spores have also been determined to survive during processing and storage of functional foods (43) thus, supporting their incorporation into extensive and novel delivery formats that do not need refrigeration. Furthermore, *B. coagulans* can metabolise a variety of plant substrates to acidic fermentation products (64, 71) making them candidates for synbiotic gut health outcomes when paired with prebiotic DF.

The prebiotic ingredients for the study were selected for having the biochemical complexity of fruit and vegetable cellular materials in order to reflect natural whole food products in contrast to fibre fractions of purified ingredients. PSCF and GBRS are each prepared using technologies that focus on minimal processing and preserving the nutritional components of the respective plant materials (72-74). Such fibres, in addition to retaining

micronutrients and polyphenols, also contain both soluble and insoluble fibre fractions that have rapid- and poor-fermentable properties at ratios that more closely represent natural whole plant foods. Whole plant PSCF has a high total DF content (87%) (Appendix I) resistant to digestion and had been determined to impart positive effects on human gut microbiota in *in-vitro* study (75). GBRS flour, produced from Lady Finger bananas (Appendix II), was also selected as a rich source of resistant starch easily fermentable as well as containing potential bioactives such as 5-hydroxytyptophan (5-HTP) (72). Resistant starch (RS) from green banana has been demonstrated to prevent intestinal inflammation (76) and modulate oxidative stress (77) in animal models of colitis and impart anti-diarrhoeal effects in children (78, 79). The study therefore used prebiotics with different fermentation properties in combination with a probiotic ingredient known to digest fibres in order to amplify potential beneficial effects owing to synergy (55).

Although, clinical trials are an integral part of validating the interventions targeted at improving human health, they do not necessarily allow understanding of the mechanism of action or the optimisation of test ingredients to achieve maximum benefits. To uncover the mechanistic functioning of synbiotic efficacy, examining affects on colonic and systemic inflammatory parameters is needed. This requires the collection of samples including colonic tissues, mucosal contents and blood through invasive procedures not generally practical in human subjects due to ethical implications. Moreover, the optimisation of compatible ingredients and the dose response information can more easily and practically be achieved using validated *in-vitro* and *in-vivo* animal models. This study, therefore, opted to screen the effective probiotic and prebiotic ingredients for synergistic synbiotic combinations using *in-vitro* and *in-vivo* mice models to better understand their mechanism of action and allow optimisation of efficacy for the development of potent synbiotic combination for human food application.

1.3 Objectives of the Thesis

The overall aim of this present study was to develop shelf stable functional foods for gut and immune health by understanding the mechanisms to promote efficacy of synbiotic combination carrying probiotic and prebiotic components in promoting gut health by mitigating gut inflammation

The detailed objectives were:

- To determine the stability and bioefficacy of probiotic *Bacillus coagulans* spores *in-vitro*

- To evaluate the individual and combined prophylactic beneficial effects of probiotic (*B. coagulans* spores), prebiotic (PSCF and GBRS) and their respective synbiotic combinations (*B. coagulans*-PSCF and *B. coagulans*-GBRS) in alleviating development of chemically-induced acute colitis in mice model of IBD

- To investigate the individual and combined therapeutic beneficial effects of probiotic *B. coagulans* spores, PSCF and their synbiotic combination in ameliorating chronic colitis in spontaneous colitic Winnie mice model of IBD.

1.4 Thesis Overview

Chapter 2 reviews the background literature associated with the known causes and treatments of IBD, and the efficacy of probiotic bacteria, prebiotics to attenuate inflammatory gastrointestinal conditions such as IBD. In addition, this Chapter also reviews the evidence for a two-point synbiotic approach to exert augmented beneficial effects to mitigate gut inflammation.

Chapter 3 evaluates the *in-vitro* ability of *B. coagulans* spores to survive simulated digestion. The ability of *B. coagulans* spores to adhere to human colonic epithelial cell lines and their immunomodulatory capacity *in-vitro* is also examined. The chapter is published in *Journal of Functional foods,* 2019 (DOI 10.1016/j.jff.2018.10.031) with minor modifications.

Chapter 4 determines the prophylactic efficacy of diet supplemented with synbiotic combination of *B. coagulans* spores and whole plant PSCF in attenuating DSS-induced acute

colitis in mice model of IBD. The individual beneficial effects of *B coagulans* and PSCF supplemented diets alone are also compared. The chapter is published in *Nutrients,* 2019 (DOI 10.3390/nu11040818) with minor modifications.

Chapter 5 contrasts the prophylactic efficacy of diet supplemented with either *B. coagulans* spores, GBRS flour or its synbiotic combination in ameliorating disease outcomes of DSS-induced acute colitis in mice in comparison to the that of more complex slowly digesting PSCF.

Chapter 6 confirms the therapeutic efficacy of diet supplemented with synbiotic combination of *B. coagulans* spores and PSCF in mitigating chronic inflammation using the different spontaneous colitic Winnie mice model of IBD. The individual beneficial effects exerted by *B. coagulans- and* PSCF-supplemented diets are also examined and compared to that of synergistic synbiotic combination.

Chapter 7 discusses the combined Thesis evidence of potential impacts of synbiotic supplementation in mitigating the inflammatory cycle in IBD owing to the synergism of probiotic and prebiotic components. The potential mechanistic functioning of synbiotic combinations in exerting augmented health outcomes are outlined and the potential for functional foods applications targeted at improving gut health in human IBD is projected.

Chapter 2
Review of the literature

2.1 Introduction

The significant role of specific dietary components in influencing either gut homeostasis or its dysfunction has long been appreciated (80). While the mechanism of diet in impacting gut health is multifactorial, its ability to govern the enteric microbiota is considered a major attribute in influencing the health and disease (9, 10, 81). High intake of diet rich in refined carbohydrates, fats, processed foods and low intake of fruits and vegetables has been linked to increased risk of gut inflammatory conditions (9, 82). IBD is a group of chronic relapsing gastrointestinal disorders that are characterized by inflammation of the GIT (19). IBD is a global disease, with over 1 million patients in USA and 2.5 million in Europe (83). IBD has emerged as a medical condition of concern in newly industrialised countries in Asia, South America and Middle East and its occurrence is rising worldwide. Australia has one of the highest incidence rates with more than 85,000 IBD patients and by the year 2022 this figure is expected to surpass 100,000 (84, 85). These data highlight the urgent need for research into prevention of IBD and innovations in health-care systems to manage this complex and costly disease.

This review encompasses the literature associated with pathophysiological features of IBD as well as applications of prebiotic DF, probiotic spores and synbiotic combinations in attenuating the gut inflammation by targeting of resolve the inflammatory features of IBD. Dietary components such as prebiotic DF and probiotics are important in the context of IBD due to their ability to modulate microbial composition and metabolites, regulate immune parameters and strengthen the colonic barrier integrity thus leading to reduced gut inflammation (19, 26, 28). The use of synbiotic formulations that capture the synergy of probiotic and prebiotic functioning is considered a pragmatic approach to resolving the gut inflammatory cycle (26, 28, 86). The IBD pathophysiological circuit features of altered barrier integrity, dysregulated immune response and gut dysbiosis are discussed in detail. The efficacies of prebiotic DF and probiotic spores in influencing the gut inflammation parameters in humans and animal studies are highlighted. The evidence for application of complex DF and stable probiotic spores for optimum benefits is also reviewed along with existing evidence

supporting the application of synergistic synbiotic combinations as a two-point approach in mitigating the inflammatory cycle in IBD.

2.2 Inflammatory bowel diseases (IBD)

2.2.1 Major forms of IBD

IBD is comprised of two major, partially overlapping but distinct, clinical entities: ulcerative colitis (UC) and Crohn's disease (CD). CD involves the entire GIT but UC is limited to the colon and rectum (19). Both diseases are characterised by a series of relapses and remissions. CD is a transmural, granulomatous condition that may involve any part of the GIT but there is a higher incidence in the ileum and the colon. In contrast, UC explicitly involves the colon of the intestine and manifests as superficial inflammation confined to the mucosal and submucosal layers of the intestinal wall (87). Unlike UC, CD is commonly associated with complications such as strictures, abscesses and fistulas. Microscopic features of UC include cryptitis and crypt abscesses while, that of CD include thickened submucosa, transmural inflammation, fissuring ulcerations and non-caseating granulomas (88). Both forms of IBD can begin relatively early in life and persist for long periods and also present as overlapping symptoms including rectal bleeding, abdominal pain, weight loss, diarrhoea and fatigue, leading to decreased quality of life (89). Additionally, both UC and CD are associated with an increased incidence of gastrointestinal cancer that has a high mortality rate (90).

2.2.2 Aetiology and pathogenesis of IBD

Although, the etiopathology of IBD remains largely unknown, emerging evidence supports the interrelated roles of genetic, environmental, microbial and immunological factors (88, 91). IBD is thought to result from an inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host (88). Alterations in the intestinal epithelial and mucosal barrier are known to support bacterial translocation resulting in dysfunctional intestinal inflammatory cascade (92-94), leading to pathologic proliferation of inflammatory mediators. There is an increasing level of evidence highlighting the key role of intestinal microbiota in driving inflammatory response during disease development and progression (95-97). The microbial imbalance in the colon, also known as gut dysbiosis, is associated with dysregulated immune responses (98) that further disturbs the colonic health. Thus, IBD encompasses a distinct tripartite pathophysiological circuit involving altered

colonic mucosal barrier, dysregulated immune response and gut microbial dysbiosis as hallmarks of this complex pathology (19).

2.2.3 Compromised colonic mucosal barrier function in IBD

The colonic mucosal barrier is composed of inner and outer mucus layers impregnated with antimicrobial factors and underlying epithelial cells stitched together with connecting protein networks called tight junctions (TJs) (99). These cells establish a barrier that separate the microbiota, food and other luminal contents from the innate and adaptive immune system, while allowing nutrient absorption and waste secretion. In a healthy gut, the continuous mucus layer in the colon keeps the surface epithelium out of contact with luminal microbiota thus, there is relatively little interaction between them maintaining the immune tolerance to the luminal microbes (19, 100). In IBD, this mucosal barrier is disrupted, resulting in translocation of the intestinal microbiota and activation of immune system leading to aggravation of the disease (100). As with dysbiosis, it is debated whether alterations noted in barrier function are the result or the causes of the disease (19).

The inner mucus layer shows increased permeability in IBD mediating interaction of the microbiota with otherwise inaccessible epithelial surfaces (101-103). The increased permeability in IBD could be attributed to several factors including altered composition of the mucus components secreted by goblet cells, reduced mucin (100), reduced glycosylation products (104), decreased trefoil factor (105) or decreased secretion of antimicrobial factors into the mucus by epithelial cells (Reg3γ), Paneth cells (defensins) and plasma cells (Immunoglobulin (Ig) A) (106-108). In UC, but not CD, the mucus layers are thinner or absent and the goblet cells responsible for mucus production are depleted (103). Mice models of UC with mutation or deficiency in major protein Muc2 have been shown to develop spontaneous colitis (109, 110) thus, indicating an important role of mucus in maintaining the barrier integrity. Certain members of the IBD-associated microbiota are known to utilise mucus as energy source and tightly regulate its production. This suggests that alterations in the mucus may be as a result of dysbiosis rather than a cause (111, 112).

The increased paracellular permeability in IBD may also be a result of abnormalities in the tight junction proteins that connect the epithelial cells (113, 114). Both environmental factors (microbes, diet) and genetic factors can influence TJ integrity (115). Compromised TJ integrity permits transit of microorganisms beyond the mucosal surface to

gain access to the immunologically active submucosa. Subsequently, the interaction of microbial components, including lipopolysaccharides, flagellin, pilli and lipoteichoic acid, with the immune system triggers the inflammatory immune response thus sustaining the disease condition (116, 117).

2.2.4 Dysregulated immune responses in IBD

The mucosal immune system orchestrates homeostasis by balancing the host response to pathogens while not responding to stimuli from commensal microbiota and dietary antigens (91). Commensal bacteria dwelling in the intestinal lumen contribute to immune tolerance. However, in IBD, their translocation to the lamina propria triggers a pro-inflammatory response leading to perturbation of immune homeostasis. The disruption of barrier function also initiates innate immune responses to the invading bacteria by innate immune cells, including macrophages and dendritic cells (DCs). Additionally it alters lymphocyte function in both the LP and mesenteric lymph nodes (MLN), and the T cell population of the normal gut mucosa (19).

Immune cells involved in the pathogenesis of IBD are the lymphocytes and antigen-presenting cells (APCs), that interact through an array of cytokines. Under normal physiological states, inflammation is regulated by a delicate balance of T-helper (Th)-1, Th17, Th2, Th3, Th9, and regulatory-T (Treg) cells (118-120). Cytokines are essential mediators of the communication between activated immune cells and non-immune cells, including epithelial and mesenchymal cells (121). A prime mediator of the intestinal inflammation is tumour necrosis factor alpha (TNF-α) which is produced by macrophages and Th 17 cells. Increased TNF-α expression has been implicated in both UC and CD (122). Consequently, TNF-α elicits expressions of Interleukin (IL)-1β and IL-6, both of which are also increased in the serum of IBD patients (123, 124). Interferon gamma (IFN-γ) is another central mediator that is elevated in IBD and associated with the severity of the disease (118, 125). Another cytokine that is closely related to disease activity is pro-inflammatory IL-8, which is found to be increased in IBD patients (126, 127). Although UC is often described as a Th2-mediated disease, CD is traditionally viewed as a Th1 condition. However, the classic paradigm has recently been changed, since cytokines can have diverse and opposing actions (128) and there are some distinct differences in the cytokine profile between UC and CD. For instance, IL-13 that affects the tight junctions and apoptosis is upregulated in UC but not CD (129, 130). IL-10 is a major anti-inflammatory cytokine that

helps maintain immune homeostasis. It has been shown to be affected in the inflamed mucosa and granulomas of CD patients (131). By inhibiting the release of pro-inflammatory cytokines and antigen presentation, IL-10 mediates attenuation of mucosal inflammation (132). Overexpression of IL-23 in the intestinal mucosa has been determined to inhibit IL-10 production. This then weakens the defensive barrier by suppressing the production of IgA in the gut (133).

Immune mediators, particularly cytokines, play a vital role in IBD, hence several current therapies are targeted towards immunosuppression and modulations of immunological pathways and responses (134). Current pharmacological treatments used in clinical practice, like thiopurines, anti-tumour necrosis factor (TNF) and corticosteroids are effective but, have limitations. The introduction of anti-TNF agents, like infliximab, into clinical UC and CD has been for both for the induction of remission and as maintenance therapy. This has markedly improved the clinical outcomes of IBD patients (135). However, these drugs are expensive and associated with unpredictable side-effects including infusion reactions, infections and lymphoma. Furthermore, nonresponse or loss of response to anti-TNF commonly occurs in CD patients, suggesting limited efficacy of the drug treatment approach (136). Research for new oral and parenteral substances regulating the alternative immune pathways like the IL-12/23 axis, IL-6, Janus Kinase inhibition and tumour growth factor (TGF)-β pathway has intensified (134). The inhibition of adhesion and migration of leukocytes into the inflamed intestinal mucosa has also received much attention. However, IBD is a very heterogenous condition, where patients have varied genetic and environmental backgrounds, and can display a wide variety of clinical phenotypes. In addition, unpredictable responses to different immune therapies makes it difficult for clinicians to choose the appropriate drug according to risk factors and clinical course (134). Moreover, cytokines have prominent and complex roles in IBD pathogenesis and the cytokine-based therapies therefore, must have higher specificity and reduced toxicity. Furthermore, the limited efficacy of the current immune-therapies in clinical practice (20) highlights the need for the developing therapies that target modulation of not, only immune responses, but subsequently mediate regulation of the epithelial barrier integrity and microbial dysbiosis, that play equal roles in the IBD pathogenesis. While, the immune system is therefore known to play a crucial role in the IBD pathogenesis, emerging evidence suggests a key role of invading gut microbiota in potentiating the pro-inflammatory immune responses (137, 138).

2.2.5 Microbial dysbiosis in IBD

Healthy gut microbiota plays a key role in metabolism of food and drugs, development GIT epithelium, development of and modulation of immune system and protection from infections (139). The microbial profile of the GIT is shaped by both genetic and environmental factors (140). Compared to that of healthy individuals, microbiota in IBD patients have been consistently shown to present as altered in the microbial composition, as well as being reduced in overall biodiversity. This is often referred to as dysbiosis (141-145). Though the key role of dysbiotic microbiota in the pathogenesis of IBD has long been perceived, the extent to which the microbial alterations are a cause or an effect of inflammation remains a debate (137).

In IBD, both expansion of potential pathogens and global changes in composition (increased or decreased abundance of indicator species) have been reported. For instance, one of the most consistent findings is a reduction in the commensal spore-forming and butyrate-producing Clostridium clusters IV and XIVa of the *Firmicutes* phylum (142, 143, 146). Also, the abundance of *Faecalibacterium prausnitzi*, also belonging to the *Firmicutes* phylum, is often reduced in the stools of CD patients (142, 147-149). However studies focused on mucosal biopsies have also contradicted this association (150, 151). The members of *Ruminococcaceae* of the *Firmicutes* phylum are also depleted in IBD, especially in ileal CD (143, 152). Other SCFA-producers including *Odoribacter* and *Leuconostocaceae* are reduced in UC, and *Phascolarctobacterium* and *Roseburia* are depleted in CD (152). *Roseburia* species belonging to the *Firmicutes* phylum are also well known butyrate producer candidates of the gut microbiota (153-155). The abundance of *Roseburia* is reduced in all IBD subgroups (152), and is connected to the family of *Ruminococcaceae* as it relies on its members to produce acetate, which it uses to produce butyrate (156). Reduction in butyrate producing bacteria is thus an important marker of dysbiotic pattern in IBD.

Conversely, members of the *Proteobacteria* phylum, such as *Enterobacteriaceae* including *Escherichia coli*, are consistently elevated in IBD patients relative to healthy individuals (143, 157). Ileal CD patients were noted to host increased proportion of adherent-invasive *E.coli* (158-160). In a study by Sasaki et al., *E. coli* isolated from UC subjects, unlike that from CD subjects, were comparatively less invasive (161). Invasive *E. coli* from UC and CD patients in their study, were shown to induce IL-8 and tumour necrosis factor (TNF)-α and reduce trans-epithelial resistance as well as stimulate disorganisation of tight junction

complexes. These observations suggest a potential pathogenetic role of adherent-invasive *E. coli* in CD. It is interesting to note however, that *E. coli* Nissle strain is used as probiotic with affirmed immunomodulatory potential (162). *Klebsiella oxytoca* is commonly linked to antibiotic-associated colitis (163). *Fusobacterium* spp., that are adherent and invasive, have been noted in higher numbers in UC subjects compared with healthy individuals (164). The invasive potential of human *F. nucleatum* correlates positively with IBD status of the host, identifying it as a potent driver of IBD pathogenesis (165). Conclusively, higher abundance of specific members of *Proteobacteria* also serves as a signature of microbial dysbiosis in IBD.

The evidence suggesting a role of *Bacteroidetes* in dysbiosis is conflicting, with some reporting their significant reduction in IBD (143, 166, 167) and during antibiotic associated diarrhoea and *Clostridium difficle* diseases (168, 169), while other studies report dominance of *Bacteroidetes* spp. in IBD (102, 170, 171). The increased prevalence of *Clostridium difficile* is also reported in IBD (172, 173). *Bacteroides* spp. and *Clostridium* spp. are the key species for generation of SCFAs in the colon. They have been reported to be diminished in IBD subjects in comparison with healthy counterparts (174, 175). The population of *B. fragilis* also has been reported to be diminished in IBD patients in comparison with healthy subjects (174). Capsular polysaccharide A from *Bacteroides fragilis* has been shown to stimulate colonic Treg cells, improving the production of anti-inflammatory cytokine IL-10 and thus providing protection against experimental colitis in mice (176). Also, mucin-degrading bacteria such as *Ruminococcus gnavus* and *R. torques* are increased disproportionately to total mucosa-associated bacteria in intestinal epithelium of both CD and UC (112). Certain bacterial species such as *Akkermansia muciniphila* and *Enterohabdus mucosicola* are also known to degrade mucus and can thrive on mucus layer (177). While, some studies reported decline of *A. muciniphila* (112, 178, 179) in IBD patients, others noted its increased abundance in rodent IBD models (180-182). However, *A. muciniphila* belonging to Verrucomicrobia phylum is abundantly present in healthy humans accounting for about 1-4% of the bacterial population in the colon (183, 184) and known to be a modulator of gut homeostasis (185). The extracellular vesicles of *A. muciniphila* were shown to protect against DSS-induced colitis suggesting its anti-inflammatory potential (186). To reach clear consensus, more well-designed clinical studies are required to analyse the roles of specific bacteria in inflammation for which confounding observations were noted.

Increased prevalence of sulphate-reducing bacteria (SRB) was also reported in IBD in conjunction with a drop in clostridia of groups IV and X Iva, especially in UC subjects (187).

SFB metabolise sulphate into hydrogen sulphides, which are detrimental to colonocytes. Hydrogen sulphides also hinders butyrate utilisation, stimulate cell hyperproliferation, deter phagocytosis and induce bacterial inhibition (188). The hydrogen sulphide producer, *Desulfovibrio* spp. have been also related to IBD (189). SRB populations, or their metabolic activities, were significantly higher in studies comparing UC patients with those of healthy controls or with UC patients in remission (190-192) as well as in the DSS mice model of colitis (193).

Inflammation by itself may be also a potent driver of the failure to resolve dysbiosis (194). Certain bacteria, including members of *Enterobacteriacea*e family are reported to better survive under the prevailing conditions in the inflamed gut, than the anaerobic commensals that dominate in healthy gut (195-198). *Salmonella enterica* serovar Typhimurium, for instance, has reduced susceptibility to host-derived antimicrobials and has developed the capacity to incorporate host compounds produced during inflammation for growth (199-201). Artis (202) postulated that elevation of potentially harmful microbial populations induces a pro-inflammatory cycle. According to this theory, opportunistic species, for instance adherent invasive *E. coli*, interact with the mucosal surface to weaken barrier integrity and trigger host immune responses against other commensals. This supports their own survival in the gut while lengthening dysbiosis. Inflammation-induced aberrancies of potentially anti-inflammatory species (such as SCFA producers) may aggravate the cycle of inflammation, concurrently favouring the growth of resistant pro-inflammatory species (203). No consistent differences between the probiotic genera *Lactobacillus* and *Bifidobacterium* have been identified (175, 204). Psychotropic bacteria including *Yersinia* spp. (205, 206) are associated with IBD, while role of *Listeria* spp. (207-210) in IBD is contradictory.

There is therefore no clear consensus on general differences between the microbiota of CD and UC and whether these changes are primary or secondary events. Some studies report similarities in CD and UC (143, 211, 212), whereas other investigators show disease-specific changes (157, 161, 170, 213, 214). Similarly, some researchers describe differences between the microbial population in active and in inactive IBD (157, 211, 215), whereas others see no difference between active and inactive IBD states (170). Studies in murine models of IBD suggest that bacterial composition changes with colonic inflammation and/or infection (196, 216-218), thus implying that the inflamed mucosa and/or altered inflammatory environment selectively affects the growth and adherence of different bacterial species. The gut microbiota of rodents, however, is not identical to that of human but conforms to the general features of

bacterial communities in the colon of the mammals. Rodent models have been instrumental in demonstrating the interaction of the intestinal microflora and the mucosal immune system (216). In spite of this care should be taken in translating the results from murine to human diseases (219). Changes in the composition of the gut microbiota can lead to alterations in the metabolites (220, 221) and have been implicated to have role in IBD pathogenesis as shown in mice models of IBD (217, 222, 223).

2.2.6 Altered metabolomic profile in IBD

Compositional and functional alterations in the colonic microbiome are likely to have significant impact on the pathogenesis of IBD (137, 146). Metabolomic studies have helped to establish the correlation between microbial composition and specific metabolic pathways thus, leading to assessment of a specific molecule products on IBD pathogenesis (137). Morgan and co-workers, compared the gut microbiota of healthy individuals and IBD patients and reported that 12% of metabolic pathways were significantly different, compared with only 2% of genus-level alterations (152). Particularly, decreased carbohydrate metabolism and amino acid biosynthesis in favour of nutrient transport and uptake was identified. Moreover, increase in virulence and secretion pathways, as well as higher expression of genes related to oxidative stress, were noted. In another study, relative abundance of bile salt hydrolase showed substantial reduction particularly among *Firmicutes* from patients with CD (224). The levels of secondary bile acids were found to be reduced in IBD patients, particularly during flares of the condition, supporting the previous finding (225). In this context, bile acid signalling has been shown to be protective in DSS and 2,4,6-trinitrobenzenesulfonic acid models of colitis by inhibiting pro-inflammatory cytokine production (226). These findings emulate the microbial responses to the inflammatory intestinal milieu of IBD and indicate that the functional and compositional differences are extremely informative when studying dysbiosis (137).

The depletion of certain bacteria and loss of their protective functions are also known to negatively affect the SCFA production. Many of the protective functions of the bacteria are associated with their ability to ferment dietary fibres to generate SCFA (227). The major SCFA in the gut are acetate, propionate and butyrate, which account for more than 95% of all the SCFA content (228). While, acetate and propionate are found in both small and large intestines, butyrate is found mainly in the colon and caecum (229). Their concentrations in the gut are typically found in a ratio of 3:1:1 (230). SCFA's particularly butyrate, are a key source

of energy for the colonocytes (231) and regulate T cell homeostasis (232). Decreased abundance of members of *Bacteroides* and *Clostridium* groups IV and XIVa (143), that preferentially produce butyrate and other SCFA, could explain the observed SCFA reduction in faecal extracts of IBD patients (146, 221). SCFA produced by these members of *Clostridia* spp. have been shown to elevate Treg cell function in the intestinal mucosa via activation of G-protein-coupled receptors (GPRs) (232). This process enhances the restoration of immune tolerance and decreases inflammation in mouse model of colitis (232, 233). Reduction in the abundance of *F. prausnitzi*, that are also major butyrate producers, is linked with high risk of postoperative recurrence of ileal CD and its administration has been demonstrated to reduce inflammation in 2,4,6-trinitrobenze sulfonic acid (TNBS)-colitic mice (147).

Beneficial effects of SCFA in IBD patients have been reported (234-237). SCFA mixture (sodium acetate, sodium propionate and sodium butyrate) enemas as an adjuvant therapy have been shown to enhance the efficacy of classic IBD treatments such as 5-aminosalicylic acid and corticosteroid therapy (238). Butyrate, acetate and propionate, by binding to specific GPRs (GPR41, GPR43, GPR109A), have been examined to benefit epithelial integrity and modulate immune response (227, 239-241). Collectively, SCFA have profound effects on the regulation of gut immunity and the pathogenesis of IBD.

Compositional and functional alterations of the enteric microbiome induce metabolic changes that can add to the toxicity of gut inflammation in IBD. Of note, fibre-rich diets are known to elevate SCFA production and have received much attention owing to their beneficial effects in reducing gut inflammation (23, 59, 77, 239). Moreover, manipulating the dysbiotic microbiota to reduce the abnormal abundance of pathogenic species and enhancing the activity of the beneficial species has tremendous potential for therapeutic benefit (145). Furthermore, therapies that function by targeting the overall inflammatory circuit seem more pragmatic over therapies that are based solely on either immunosuppression or by influencing microbiome function. In this context dietary ingredients, such as prebiotic DF and probiotics, that function by influencing the gut microbiota composition and associated metabolic functions as well as regulating the immune system and barrier integrity, are thought to be useful in mitigating inflammatory circuit leading to resolution or prevention of IBD.

2.3 Dietary interventions in IBD

Despite the pharmaceutical approaches to disease control having improved in recent years, the limited efficacy and side-effects of the current immune-therapies in clinical practice (20) has highlighted the need for more research and development of alternative, safe and effective treatments. The current IBD paradigm focusses on three main areas: the microbiota and metabolites, the mucosal barrier and the immune system (19). Research findings have shown the impact of dietary components such as prebiotic DF and probiotic on all three components the IBD paradigm (23, 25, 26). Prebiotic DF and probiotic are developing as preventive and corrective treatment therapies for IBD (242) by aiming to reduce gut inflammation and thus consequently helping to prevent relapse.

2.3.1 Prebiotic Dietary fibre approach in attenuating IBD

2.3.1.1 Implications of low-fibre diet in IBD

Besides the role of genetic predisposition in the development of IBD (88), diet is a major factor affecting the enteric microbiota. Numerous studies have considered the role of specific dietary components in the development of IBD and the in the course of the disease (9, 23). The trend towards "Westernised-diet", characterised by high dietary intake of refined carbohydrates, animal proteins, ultra-processed foods and low intake of fruits and vegetables rich in DF, has been linked with increased risk of both CD and UC (82). A systematic review of literature by Hou et al. (9) concluded that a high intake of total fats, polyunsaturated fatty acids, omega-6 fatty acids and meat were consistently associated with increased risk of developing UC and CD. High vegetable intake was also consistently linked with reduced risk of UC, whereas fibre and fruit intake were consistently associated with decreased risk of CD.

DF is a vital component of diet in the context of IBD. A protective effect of a high-fibre diet on the intestinal disorders was pointed out by a study that observed a low incidence of colon cancer and other non-infectious intestinal diseases among the populations of African countries, whose diet was typically rich in DF (80). Another study investigating and comparing the human gut microbiota from children, characterised by a modern western diet in European children and a rural high-fibre diet in African children, revealed distinctly increased microbial diversity in African communities with high abundance of bacteria of genus *Prevotella* capable of metabolising DF, and low abundance of certain pathogens and high SCFA levels (10). The recent follow-up study by same researchers demonstrated the effects of

this dietary switch on the gut microbiota of children (81). Their findings clearly illustrated that the gradual increase of animal proteins (meat and dairy products), saturated fats, and processed and refined foods to the rural vegetarian diet of urban African children markedly altered their microbial profiles and gut functioning with, concomitant reduction in microbial population able to ferment DF and a sharp rise in other bacterial groups with capacity to metabolise animal proteins, animal fats and sugars. The authors concluded that dietary habit modifications, in the course of urbanisation, play a role in shaping the gut microbiota, and that prior fibre-degrading bacteria are at risk of being eliminated by the advent of westernised lifestyle. However, it is commonly observed that IBD patients are permanently on low fibre diets, regardless of disease activity (243, 244). Continuation of a regular diet is recommended during mildly to moderately active disease states, both in UC and CD patients by the European Crohn's and Colitis Organisation. The fibre-restricted diet should always be used on a temporary basis and is indicated in few cases including, acute relapse (with diarrhoea and cramping), intestinal stenosis, and small intestinal bacterial overgrowth and some post-surgery instances (23, 245). Slow and gradual reintroduction of high-fibre foods is recommended during the periods of no symptoms and no or mild disease activity (23, 246).

2.3.1.2 Prebiotic dietary fibre – a definition

The majority of DF originates from plant cell walls (PCW) that are key in maintaining plant structure and function. Chemically DF is mostly comprised of carbohydrate polymers that resist digestion in the mammalian small intestine but undergo fermentation by bacteria in the colon (52). According to the American Association of Cereal Chemists (AACC) (247), DF is "the edible parts of the plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine, with complete or partial fermentation in the large intestine". Based on this AACC report of 2001, DF promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation. In compliance with this definition DF includes non-starch polysaccharides (NSP) and resistant oligosaccharides (e.g. cellulose, hemicellulose, pectin), analogous carbohydrates (e.g. resistant potato dextrins), lignin, and substances associated with NSP and lignin complex in plants (e.g. waxes, cutin) (247). CODEX Alimentarius commission in 2009 denoted DF as carbohydrate polymers with ten or more monomeric units, which are neither digested nor absorbed in human small intestine and belong to the following categories: edible carbohydrate polymers naturally occurring in the food consumed; carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means that have been shown to

have physiological benefit to health; or isolated or synthetic fibres that have been shown to have physiological benefit to health (248).

Some dietary fibres, such as inulin-type fructans and galacto-oligosaccharides, are also categorised as prebiotics. Prebiotics were defined by Gibson et al. as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or limited number of bacteria in the colon and thus improve host health" (24). The selection criteria for a compound to be recognised as prebiotic include: "(i) resistant to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption, (ii) fermentation by intestinal microbiota, and (iii) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing" (249). In 2015, Bindels et al. (51) proposed that an updated definition highlighting the influence on overall microbial diversity as the key factor rather than targeting specific species and defined prebiotic as "a non-digestible compound that, through its metabolization by microorganism in the gut, modulates the composition and/ activity of the gut microbiota, thus conferring a beneficial physiological effect on the host". This definition emphasised the physiological effects of metabolites that result from fermentation, especially SCFA's. However, it limited prebiotics to interactions with gut microbiota (with microbial fermentation as the key to the prebiotic concept) and excluded extraintestinal sites such as vagina and skin (250). Although fructo-oligosaccharides, galacto-oligosaccharides and lactulose fulfil all the prebiotic definition requirements, several other dietary carbohydrates, as well as polyphenols and polyunsaturated fatty acids converted to respective conjugated fatty acids, are also potential prebiotic candidates assuming convincing weight of evidence in the target host (249, 250).

2.3.1.3 Types of dietary fibre

From the physiological point of view, the two main types of DF are water soluble (e.g. fructans, pectin, β-glucan) and water insoluble (e.g. cellulose, some hemicelluloses, lignin) DF. Despite the fact that the solubility is not always associated with a particular physiological effect, as pointed out by the experts of the Food and Agriculture Organisation of the United Nation/World Health Organisation (FAO/WHO) in 1998 (251), this division is still used and current (23). DF can be then divided into fractions that are rapidly fermented (e.g. oligosaccharides), slowly fermented (e.g. gums) and those that are hardly fermented (e.g. wheat bran). Soluble DF is a type of fibre that dissolves in water and includes pectins, arabinoxylans, glucans and specific types of gums (52). It attracts the water to form a viscous

gel depending on its chemical structure and molecular weight. Soluble DF tends to slow the colonic transit time and nutrient release as well as inhibit the action of α-amylase (52, 252). This in turn, can lead to a reduced glycaemic response, thus regulating blood glucose (253, 254). The degree to which PCW polysaccharides can be fermented varies considerably, with lignin that is considered insoluble, being very resistant to fermentation, and pectin, which is highly soluble, usually being fermented completely.

Generally soluble fibres are assumed to be fermented more rapidly than insoluble fibres in the colon (255-257) although this perception is changing (258, 259). The most active sites are the caecum and proximal colon where most carbohydrates disappear (256). The types of DF that reach the colon, however, have significant implications for the site of its degradation. The mean transit time of the caecum is only 1-3 hours and the main NSP degraded at this site are soluble fractions (e.g. pectin, β-glucan, soluble arabinoxylans etc) (260). Depolymerisation of soluble fractions, as well as swelling and high water binding capacity, are some of the factors that encourage easier microbial colonisation and degradation of these substrates (261, 262).

Insoluble fibres pose a significant challenge to gut microbiota owing to its reduced surface area and the hydrogen-bonding networks that hold the carbohydrate chains together (263). Insoluble fibre fractions may also be extensively degraded during the passage of the colon. For instance, a linear xylan present in the aleurone cells of rye was shown to be slowly, but completely fermented. In contrast, cellulose, arabinoxylans and xylans, when present in lignified tissues, are more resistant to degradation in the colon (260). Common insoluble DF include cellulose and lignin. Williams et al. (52), noted, that there is no standardised method for separating soluble and insoluble fibres and conditions used may differ in term of temperature, solvents and fibre to solvent ration. All of these govern the partition of fibre materials into soluble and insoluble fractions, hence, so the categorisation has substantial limitations.

Non-fermentable water-insoluble fibres increase the volume of the stool, and by mechanical stimulation/irritation of the gut mucosa also reduce faecal transit time (23). Fermentable insoluble fractions, such as some forms of resistant starch (RS), undergo partial fermentation by the gut microbiota. RS is the sum of starch and starch degradation products that are not digested and absorbed in the small intestine and reach unaltered into the colon (264). RS is categorised into 5 types (RS1-5). It offers some of the benefit of the water-

insoluble fibre and some of the benefits of water-soluble fibre. SCFA's produced via bacterial fermentation are frequently cited as the important contributor to the beneficial effects of DF (265).

Moreover, certain polyphenols present in fruits and vegetables, ingested as a part of the diet, have been shown to impart anti-oxidant and anti-inflammatory effects and hence, are of considerable interest for prevention and treatment of colitis (52, 266). A number of dietary polyphenols, including those from berries, green tea and curcumin, have been extensively tested and shown to alleviate chemically-induced colitis in rodent models in a dose-dependent manner (266). Moreover polyphenols, upon reaching colon, are known to interact with gut microbiota thus, influencing the gut microbial diversity (267, 268).

2.3.1.4 Efficacy of dietary fibre in resolving gut inflammation in IBD

While the underlying protective mechanisms of DF are likely to be multifactorial, the production of SCFA via bacterial fermentation is frequently cited as a major contributor to the beneficial effects of DF (23). Of the SCFAs produced, butyrate is the one that serves as major source of energy for colonocytes, mediating improvement of villi growth and crypt development. It also contributes to mucin production, improvement of intestinal barrier via tight junctions and stimulates production of Treg cells and anti-inflammatory cytokines (232, 269, 270). Butyrate has also been shown to stimulate peroxisome proliferator-activated receptor (PAPRγ) (271). PAPRγ antagonises several pro-inflammatory pathways such as STAT, AP-1 and NF-κB thus, its activation mediates anti-inflammatory mechanism involved in the prevention of inflammatory and immune-mediated diseases (272).

In addition to providing energy to intestinal epithelial cells, and regulating mucosal and systemic immune responses (265, 270), the production of SCFA's lowers the luminal pH that helps in influencing the composition of gut microflora by curtailing potentially pathogenic disease-causing bacteria (e.g. *Bacteroides* and *C. difficile*) and stimulating growth of protective phenotypes (e.g. *Firmicutes, Bifidobacteria* and *Lactobacillus)* (270, 273, 274). Some beneficial bacteria like *Bifidobacterium infantis* have certain enzymes that hydrolyse saccharides, resulting in their own proliferation (275). Other microorganisms can support the growth of some beneficial bacteria by cross-feeding mechanisms. *B. longum,* for instance,

releases free fructose during oligofructose degradation that supports the proliferation of other organisms that are incapable of fermenting oligofructose themselves (276). Other mechanisms for DF in influencing health include dilution of toxins via stool bulking, improvement of the colonic barrier function, modulation of microbiota and regulation of mucosal and systemic immune response (23, 277). Moreover, DF's are also known to exert anti-adhesive effects by inhibiting colonisation of pathogens to gut epithelium. Inulin has been shown to inhibit the *in-vitro* intestinal colonisation of *C*. *difficile* (278), an organism implicated in IBD that can cause nosocomial diarrhoea, colitis and even death particularly after antibiotic treatment (279). Thus, DF may influence the immune system and gut microbiota directly or indirectly via bacterial fermentation.

Evidence from rodent experimental models of IBD have suggested significant ability of DF to alleviate colitis. A number of experimental colitis studies (Table 2.1) have reported that prebiotic DF consumption is associated with reduction in the severity of inflammation, stimulation of immune responses and modulation of gut microbiota composition. Some studies advocate the application of combination of DF to exert supplementary effects owing to possible multiple mechanisms of action. Kleessen et al. (280) compared the dietary supplementation of inulin, fructo-oligosaccharide (FOS) or the combination of both prebiotics in gnotobiotic rats colonised with human enteric bacteria. FOS was found to effectively stimulate *Bifidobacterium* and *Lactobacillus* growth while inulin significantly increased luminal butyrate concentration. The mix of fructans stimulated the levels of *Lactobacilli* spp. and *Clostridium coccoides-Eubacterium rectale* cluster. The results correlate with that of other studies which reported complementary benefits in reducing inflammation when prebiotic combinations were used (274, 281). Prebiotic DF therapies aimed at modifying gut environment should be given early in the course of disease as evidenced from pre-treatment in DSS-induced colitis mice models (239, 282). Kiwifruit is a rich source of plant secondary metabolites such as ursolic acid, carotenoids, and a range of polyphenols (283). Kiwifruit extracts have been demonstrated to inhibit cytokine production by LPS-stimulated macrophages and epithelial cells isolated from IL-10 gene deficient colitic mice (284), and by influencing immune signalling pathways and metabolic processes within the colonic tissue thus decreasing disease activity (285).

A germinated barley foodstuff (GBF) prepared from brewer's spent grain by physical isolation (milling and sieving) has been demonstrated to attenuate mucosal damage in DSS-induced colitis rodents (286-288) and in UC patients (287, 289-292) (Table 2.2). The GBF

was comprised of glutamine-rich protein and the hemicellulose-rich fibre. GBF was reported to support maintenance of epithelial cell populations, facilitate epithelial repair, suppress pro-inflammatory NF-κB-DNA binding activity with increased SCFA production supporting the *Bifidobacterium* population, thereby preventing experimental colonic injury (287). The authors also reported that the low-lignified hemicellulose fibre fraction also modulated stool water content by its large water-holding capacity.

Table 2.1. Beneficial effects of prebiotic dietary fibres in rodent models of IBD

Prebiotic dietary fibre -dose	**Rodent IBD model**	**Effects/outcomes**	**Reference**
Inulin – 8 g/kg diet and **FOS –** 8 g/kg diet	HLA-B27 transgenic rats	• Reduced chronic intestinal inflammation • Increased numbers of *Bacteroides* and *Prevotella*, decreased *Clostridium* cluster XI • Reduction in colitis • Increased *Bifidobacterium* spp. decreased *Enterobacteriaceae* and Clostridium cluster XI and *C. difficile* toxin B	(274)
Inulin and oligofructose (Synergy1) (1:1) – 5 g/kg in drinking water	HLA-B27 transgenic rats	• Reduced colitis severity • Decreased in IL-1β and increased TGF-β concentrations • Increased *Lactobacillus* and *Bifidobacterium* • counts	(281)
Inulin – 1 % in drinking water, or 400 mg/kg diet	DSS-induced rats	• Reduced colitis severity • Increased *Lactobacilli* counts	(293)
FOS – 1 g/day	TNBS-induced rats	• Reduction of inflammation • Reduced MPO activity and caecal pH • Increased lactate and butyrate concentrations • Increased LAB counts	(294)
FOS – 1.5 g/mL in drinking water	DSS-induced mice	• Reduced severity of colitis • Reduced damage to distal colon • Increased crypt depth and area	(295)
FOS – 75 mg/day	Lymphocyte-driven CD4+ CD62L+ T cell transfer colitic mice	• Amelioration of colitis • Reduced MPO and alkaline phosphatase activity, lowered proinflammatory cytokine (IFN-γ, IL-17, and TNF-α) • Increased colonic Occludin expression	(296)
FOS – 63 g/kg diet **RS 3** – 115 g/kg diet	DSS-induced rats	• No reduction in severity of colitis • Higher amounts of butyrate in distal colon • Reduction in severity of colitis • Higher amounts of butyrate in caecum	(297)

Lactulose – 0.06 % in drinking water	IL-10 gene-deficient mice	• Stimulated growth of *Lactobacillus* spp. • Reduced levels of adherent and translocated aerobic bacteria	(298)
Lactulose – 300-100 mg/kg diet	DSS-induced rats	• Ameliorated DSS-induced colitis in dose dependent manner	(299)
GOS – 4 g/kg diet	TNBS-induced colitis rats	• No reduction in severity of inflammation • Increased total bacteria and bifidobacteria in faeces	(300)
RS – 20 g/100gm diet	DSS- and AOM-induced colitis-associated colorectal cancer in rats	• Increased *parabacteroides, Barnesiella, Ruminococcus, marvynbryantia* and *Bifidobacterium* • Increased SCFAs and reduced colitis severity • Reduced pro-inflammatory cytokines (COX-2, NF-kB, TNF-α and IL-1β)	(301)
RS 2 – 10 g/100g diet	DSS-induced mice	• Reduction of severity of colitis • Declined levels of *Clostridium coccoides, Enterococcus spp*. and *E. coli*	(302)
Pectin – 100 g/kg diet	DSS-induced mice	• 20-day pre-treatment attenuated clinical & inflammatory parameters • Improved anxiety-like behaviour	(282)
Pectin – 5 g/100g diet	DSS-induced mice	• Increased Ig A and decreased IgE levels in MLN and faeces	(303)
GBF – 100 g/kg diet	DSS-induced rats	• Attenuated clinical signs • Reduced serum α-$_1$ acid glycoprotein • Increased caecal butyrate • Accelerated colonic epithelial repair	(286)
GBF – 100 g/kg diet	DSS-induced mice	• Reduced disease activity and prevented body weight loss • Reduced serum IL-6 level and histological damage to mucosa • Suppressed mucosal STAT3 expression and NFκB activity • Elevated caecal butyrate and lowered bile acid	(288)
GBF – 100 g/Kg diet	DSS-induced rats	• Prevented bloody diarrhoea and mucosal damage • Elevated faecal acetate and butyrate levels • Increased the numbers of *Eubacteria* and *Bifidobacteria*	(304)

There are only a few studies investigating the DF intervention in IBD patients (Table 2.2) and these have generally presented conflicting results. Lindsay et al. (305) reported excellent efficacy of supplementation of FOS as a 15g dose for 3 weeks in reducing disease activity index (DAI) in moderately active CD patients. In a separate work, supplementation of 15 g of FOS for 4 weeks by Benjamin et al. (306) however, did not prove clinically beneficial in improving CD based on DAI. Moreover, the microbiome of treated subjects showed no differences in the faecal numbers of *Bifidobacterium* or *F. prausnitzii* from baseline. It is critical to note that the study in which FOS failed to improve DAI (306) included patients

with documented diagnosis of CD for at least 3 months while, the earlier study that showed improvement with prebiotics (305) was in recruited patients with moderately active CD. This implies the importance of application of prebiotic during the onset of disease to harness maximum benefit. Oat bran, which is a rich source of β-glucans, has also been shown to benefit patients with inactive UC (307). This 12-week pilot randomised control trial showed a 36% increase in the faecal butyrate level at 4 weeks and improvements in abdominal pain or gastroesophageal reflux after oat bran intervention. In humans, combinations of DF have also proved successful in modulating gut microbiota and improving disease symptoms in IBD patients (308, 309). Another randomised crossover trial with UC patients compared the effects of three 7-day supplement periods of fructans (oligofructose as Raftilose P95®, RS and digestible carbohydrate intake) with a glucose placebo (310). In all these patients, fructans supplementation had a fermentation ability of 83% and was associated with an elevation in faecal butyrate excretion relative to RS supplementation which had a 46% fermentation ability and resulted in elevated faecal isobutyrate and isovalerate excretion. This suggested that different DF's vary in their fermentation ability and different SCFA can be produced depending on the type of DF. Similarly, wheat bran intake in inactive UC children was more effective in reducing bile acid concentration than a psyllium supplement (311). The reduction in specific bile acids in this study was beneficial in patients as these are reported to induce a proliferative response in the gastric mucosa of rats. Bile acids may also increase mucosal cell proliferation in other parts of GIT (311). Hemicellulose-rich GBF feeding to UC patients was shown to increase stool butyrate concentrations and luminal *Bifidobacterium* and *Eubacterium* levels (292) leading to disease improvement with clinical and endoscopic scores as markers (289, 292, 312). GBF supplementation, along with standard drug therapy in mild to active UC patients, has been confirmed to reduce serum levels of TNF-α, IL-6 IL-8 (290) and C-reactive protein (CRP) (291).

Owing to the conflicting outcomes of the human studies, a single generalisation cannot be made as to the benefits or otherwise of the currently applied prebiotic DF's. However, given the complex nature of DF and varying biochemical structures of different plant materials, different studies comparing different fibres could significantly influence the varying outcomes in each study. Furthermore, results are likely to be affected by the dosage and length of time of DF intake as well as the disease type (UC or CD) and disease state (active or inactive). Nevertheless, the abilities of DF to modulate immunological parameters

and to modulate gut microbiota, which may be associated with health benefits in patients with IBD, supports their application in resolving gut inflammation.

Table 2.2. Beneficial effects of prebiotic dietary fibres in clinical IBD studies

IBD subject types	Prebiotic dietary fibre and dose	Participants (treatment/ control) and Duration of Study	Effects/outcomes	Reference
Active CD patients	**FOS –** 15 g/day	*n*= 10 adults (10/0); 3 weeks	• Increased faecal *Bifidobacterium* counts • Improved disease activity • Increased IL-10 dendritic cells	(305)
Active CD patients	**FOS –** 15 g/day	*n*= 103 adults (54/49); 4weeks	• No significant clinical benefits • No differences in faecal numbers of *Bifidobacterium* or *F. prausnitzii* • Increased IL-10 dendric cell staining and reduced IL-6 positive dendritic cells	(306)
Inactive and mild-moderately active CD patients	**Oligofructose -enriched inulin (OF-IN) –** 10 g twice/day	*n*= 45 adults (25/20); 4 weeks	• Improvement in disease activity in active CD patients • Reduced faecal *Ruminococcus gnavus* and increased *B. longum* counts • No effect on *F. prausnitzii*	(308)
Inactive CD patients	**DF-rich unrefined carbohydrate diet** (including wheat bran), NA	*n*= 7 adults (4/3); 2 weeks	• Diet consumption was feasible • No adverse effects • Improved quality of life and gastrointestinal functions • No significant differences between groups in the inflammatory markers	(246)
Active UC patients	**OF-IN –** 4g thrice daily with standard drug therapy	*n*= 19 adults (10/9); 2 weeks	• Reduced calprotectin at day 7 • Reduced dyspeptic symptoms • No changes in human faecal DNA concentration	(309)
Inactive and mild-moderately active CD patients	**OF-IN –** 10 g twice/day	*n*= 56 adults (231/25); 4 weeks	• Increased relative faecal acetaldehyde and butyrate levels	(313)
UC patients with ileal pouch	**FOS and RS** – 14.3 g/day	*n*= 16 adults (16/0); 3-period crossover/3, 7-daysupplement periods (7-day washout period)	• 83% fermentability recorded for FOS and 46% for RS • RS increased faecal excretion of butyrate • FOS reduced excretion of isobutyrate and isovalerate	(310)

Active UC and CD patients	**Lactulose –** 10 g/day	UC: *n*= 14 adults (7/7); 4 months CD: *n*= 17 adults (8/9); 4 months	• No clinical benefits observed in terms of clinical activity, endoscopic score or immunohistochemical parameters	(314)
Inactive UC patients	**Oat bran –** 60 g/day	n= 19 adults (19/0); 3 months	• Increased faecal butyrate • Reduced signs of colitic relapse • Reduced abdominal pain and gastroesophageal reflux	(307)
Mild-moderately active UC	**GBF –** 30 g/day thrice daily	*n*= 10 adults (10/0); 4 weeks	• Improved clinical and endoscopic parameters	(315)
Active UC patients	**GBF –** 20-30 g/day	*n*= 18 adults (11/7); 4 weeks	• Decreased clinical activity index scores • increased faecal counts of *Bifidobacterium* and *Eubacterium limosum*	(292)
Mild-moderately active UC patients	**GBF –** 20-30 g/day along with baseline treatments	*n*= 21adults; 24 weeks	• Decreased clinical activity with reduced visible blood in stools and nocturnal diarrhoea	(312)
Active UC patients	**GBF –** 30 g/day thrice daily	*n*= 10 adults; 4 weeks	• Clinical and endoscopic improvements • Increased stool butyrate level	(289)
Inactive UC patients	**Psyllium Husk –** 3.52 g/day	*n*= 29 adults (16/13); 4 months	• Reduced gastrointestinal symptoms: of abdominal pain, diarrhoea, loose stools, urgency, bloating, incomplete evacuation, mucus and constipation	(316)
Inactive UC patients	**Psyllium seeds** (including husk) combined with mesalamine and placebo of mesalamine alone and 10 g sachets of Psyllium twice/day	*n*= 102 adults (30/37/35); 12 months	• Both failure and continued remission of similar approximation 30% in psyllium + mesalamine 35% mesalamine 40% psyllium	(317)
Mild-moderately active UC patients	**GBF –** 30 g/day thrice daily with standard drug therapy	*n*= 41 adults (20/21); 2 months	• Reduced serum levels of TNF-α, IL-6 and IL-8	(290)
Mild-moderately active UC patients	**GBF –** 30 g/day thrice daily with standard drug therapy	*n*= 41 adults (20/21); 2 months	• Reduced abdominal pain and cramping • Reduced serum CRP	(291)

NA: not applicable

2.3.1.5 Purified versus whole-plant complex dietary fibres

Whole plant sources including fruits, vegetables, and grains are natural sources of DF. However fibre supplements that are composed of isolated or extracted fibres (using chemical, enzymatic or aqueous processes) from natural sources are also widely available and are considered as a subgroup of functional fibres (23). The most common sources of fibre in available supplements are natural, including psyllium, glucomannan, inulin, wheat dextrin, or flax, and synthetic, such as methylcellulose and calcium polycarbophil. With the increased consumer perception of beneficial effects of DF, much research has focussed on the testing of single purified sources of DF's. these are usually extracted from plants, sometimes by the use of harsh chemical procedures (52). A number of studies have been performed to examine the effect of various purified DF including polydextrose and soluble maize fibre (318), maize, dextrin, pullulan, RS (257), aloe vera gel extract and powder, Larch extract, *Undaria pinnatifida* fucoidans, Tragacanth gum, ghatti gum (319), amylose, amylopectin, dextran, xylan, polygalacturonate, pectin (320), apple pectin (321) and high amylase maize starch (322) on human gut microbiota. However, when, determining the specific effects of purified fibres on health, particularly on the microbiota, the limited biochemical complexity of the purified ingredients, in contrast to that of naturally occurring in fruits and vegetables, cannot be overlooked. The actual biochemical complexity of naturally occurring DF such that in fruits and vegetables is being recently recognised and appreciated as a vital attribute in influencing the gut microbial complexity (52-54).

The most natural form of DF consumed is as PCW's from cereals, fruits, vegetables and other-plant based foods. In this material soluble polysaccharides are typically present alongside insoluble cellulose in an hydrated but insoluble form (52). Purified fibre ingredients do not accurately duplicate the soluble to insoluble fibre ratios of natural plant sources. This differences can affect the fermentation time and rates in the colon. For instance, in an *in-vitro* study, which compared more complex arabinoxylan with purified arabinoxylan, showed that purified form had a much faster rate of gas production, and was fermented to a greater extent (323). Separate work comparing fermentation of inulin with arabinoxylan oligosaccharides noted that inulin rapidly fermented and led to microbiota changes in proximal colon, whereas arabinoxylan oligosaccharides were fermented in the distal colon of the *in-vitro* model (324). Moreover, isolated DF fractions tend to ferment much faster than when consumed as complex structures from whole plant foods (325) and thus ultimately differently affect specific areas of the GIT. PCW complexity and structure can slow fermentation by restricting enzyme

accessibility thus modulating microbial activity. These findings indicated more slowly fermentable substrates will be more likely to ferment for a longer trajectory within the colon, compared with more rapidly fermented substrates (326). DF that can ferment more slowly, and for a longer trajectory within the colon, is likely to be more beneficial in IBD by providing a trophic effect of the fermentation products (SCFAs) to the entire length of the colon while suppressing rapid gas production that can lead to abdominal cramps.

Despite the appreciation of biochemical complexity of DF to influence colon health, only a few studies (76, 77) have investigated the effects of DF functional products that closely resemble whole-plant sources, prepared with minimal processing to retain fibre complexity and other bioactive compounds, on outcomes of disease in IBD. This emphasises the need for efforts to develop functional DF supplements based on natural foods which have the carrying plant cell wall complexity of the intrinsic DF and other bioactive compounds. In this context, green banana flour, which is a rich source of RS, has been shown to have potential in ameliorating experimental TNBS-colitis in mice (76, 77). The benefits of green banana flour, owing to RS content, was associated with increased SCFA levels, increased mucin production and reduced oxidative stress in the colon. The green banana based-diet has been demonstrated to impart anti-diarrhoeal effects in humans (78, 79). The anti-diarrhoeal action is postulated to be mediated by its high content of amylose-RS, that elicits SCFA production in colon, thus, stimulating colonic salt and water absorption (327, 328). RS-rich flour, produced from green lady finger bananas, been reported to retain a high content of RS as well as vitamins and minerals (72). Moreover, this particular GB flour is reported to contain 5-hydroxytryptophan (5-HTP), a serotonin precursor, which in a separate work, has been shown to alleviate mood disturbances in DSS and TNBS mouse models of colitis (329).

Sugar cane fibre has also been reported to preserve the cell wall components and retain other intrinsic nutritional bioactive components (73, 74). It can be prepared by wet diffusion of the stem to remove most of the sucrose from the cut cane, which is then dried and ground to a flour. Such fibre, in addition to retaining other micronutrients and polyphenols, also contains both soluble and insoluble benefits as well as rapid- and slow-fermentable fibres and at ratios that more accurately represent those present in other natural whole plant foods (330). In a recent *in-vitro* study (75), this sugar cane fibre product has been shown to impart positive effects on human gut microbiota, and be effective in maintaining the microbial diversity compared with other fibres tested. The sugar cane fibre in this study was also reported to contain the highest total dietary fibre content, polyphenol and antioxidant potential compared

to other commercial fibre products tested (i.e. Benefiber®–wheat dextrin and Macro Organic Psyllium husk). Previous studies have reported sugarcane to be rich in cellulose, hemicellulose (331, 332), contain a range of β-1, 4, and α-1, 4 linkages between glucose, xyloglucans, xylans, glucomannan, arabinoxylan, glucuronoxylan and D-galacturonic acid (333, 334). Thus, fibre supplement from sugar cane stems has a complex biochemical fibre network similar to that of other whole plant foods. It could be used as a potential DF to impart prebiotic effects in gut inflammatory conditions but more studies on complex DF supplements, with biochemical fibre complexity similar to that in fruits and vegetables, is required to appreciate their benefits in IBD. Dysbiotic microbiota metabolise DF differently to the microbiome of healthy subjects and can consequently affect the levels and types of metabolites produced (335). Since, gut microbial dysbiosis is a hallmark of IBD, application of probiotic microorganisms to repopulate the gut with beneficial microbial phenotype, seems to be a pragmatic approach to enable maximum DF benefits to be attained.

2.3.2 Probiotic approach to ameliorating IBD

The mounting evidence that has drawn correlations between certain intestinal bacterial flora types and IBD pathogenesis, has resulted in many attempts to modify gut microflora by administering probiotics (242, 336, 337). The first ever study that administered probiotic to inactive UC patients was in 1917. It showed promising results in maintaining remission by application of *E. coli* Nissile (338). Probiotics subsequently been mentioned by many reviewers as a promising therapy for IBD (339-343). Multiple mechanisms have been suggested to explain the protective effects of probiotic against intestinal inflammation. Probiotics have the potential to directly or indirectly resolve the tripartite pathophysiological circuit in IBD. Mechanisms of probiotic benefits may include manipulation of intestinal microbiota, pathogen suppression and/or exclusion, immunomodulation, induction of epithelial cell proliferation and strengthening the intestinal barrier (344). The success of probiotics however, is largely dependent on the species and strains used (31). It is evident that not all probiotics are equally beneficial as each may have an individual mechanism of action and the host characteristics may determine which probiotic species and strains could be effective (32-35).

2.3.2.1 Current probiotic delivery foods and associated challenges

There is a growing demand for probiotics among consumers based on their perceived health benefits. In the food sector, incorporating probiotics into variety of food matrices to provide added health benefits beyond their basic nutrition is a global trend (336). *Lactobacillus* and *Bifidobacterium* species are well established, globally accepted and the most consumed probiotic species for their potential health benefits (345). Other preferred probiotics include strains of *Streptococcus*, *Enterococcus*, *Saccharomyces* and *Bacillus* species (43). However, certain strains of commonly applied PB belonging to *Lactobacillus* and *Bifidobacterium* species, are reported to be sensitive to gastric transit (38). Despite their ability to confer health benefits, not all of these microorganisms can be successfully administered as probiotics through pharmaceutical supplements or functional foods owing to their inability to resist hostile conditions during gastric transit or withstand harsh manufacturing and storage conditions (346).

In addition to their therapeutic application as pharmaceutical supplements, probiotics are being incorporated into a variety of food products. Probiotic preparations must meet strict criteria related to quality, safety and functionality (347). A key quality criterion is that they contain accurately defined numbers of viable cells as expressed on the product label. Some investigators reported significantly lower levels of bacterial numbers than stated (348-352), as well as inconsistency in their probiotic properties when applied in some commercial food and pharmaceutical products. This included reduced ability to survive simulated digestion and decreased adherence to human intestinal epithelial cells (349, 352). Currently, refrigerated dairy products that carry conventional PB's such as *Lactobacillus* and *Bifidobacterium* strains, are the major delivery forms for probiotic food. The colder storage temperatures and rich growth media assist retention of viability for these conventionally used probiotic strains (353). Some *Lactobacillus* strains are known to lose their viability by the end of the shelf life in cold storage even in dairy products (348, 351) thus, limiting their full probiotic potential. Lactose intolerance, high fat and cholesterol, milk allergies and also the growing trend towards vegetarianism have led to the demand of non-dairy probiotic foods (354). Moreover, probiotic-supplemented foods that have an extended shelf life without refrigeration are more convenient. Efforts are therefore being made to improve viability of PB during manufacture and storage of foods in order to deliver efficacious probiotic dose in convenient forms other

than in a dairy food base. In this context, *Bacillus* species, due to their ability to form spores, offer potential advantage over other conventionally used PB in terms of their application in probiotic supplements and functional foods (64, 355).

Spore based probiotics, being inherently heat-stable, makes them an excellent choice as a functional ingredient to be incorporated into food that requires heat application during manufacture. The spores can maintain viability and thus efficacy after incorporation into food products during manufacture. The spore supplemented food products can be stored at room temperature without any deleterious effect on the viability. Moreover, spores have the capacity to tolerate the low gastric pH (356) unlike, in some *Lactobacillus* and *Bifidobacterium* strains (357, 358), thus ensuring delivery to the colon intact of the entire effective dose of ingested bacteria. These attributes not only offer an opportunity to carry probiotics through acidic foods such as yoghurt or fruit juices, but also opens avenues for incorporating probiotic spores into shelf-stable food systems such as snack bars, baked goods, breakfast cereals and chocolates without affecting cell viability during non-refrigerated storage. This is not possible with conventional PB's. Also, with increased demand for dairy-free milk products, *Bacillus* spores can also be applied to develop probiotic non-dairy drinks. Thus, the robustness of spores, when coupled with health-promoting attributes, makes them an attractive functional ingredient in food for improved gut health in humans.

2.3.2.2 Bacillus as probiotic

Bacillus species are spore-forming, Gram-positive aerobic bacteria common in soil, water, dust and air. These microorganisms enter the gut by association with food (359). *Bacillu*s species have the capacity to form spores when growth conditions are unfavourable and can exist in the dormant stage for many years. However, favourable conditions, such as specific nutrients, pH, temperature and moisture, can trigger germination of spores into vegetative cells (360, 361). *Bacillus* species have been used as probiotics for at least 50 years with the Italian product known as Enterogermina® registered 1958 in Italy as medicinal supplement (355). *Bacillus*, as a source of probiotics, has sparked scientific interest only in the last 15 years with just a few principal reviews in the area (355, 359, 362, 363).

Bacillus spores are regularly consumed by humans unintentionally through fermented foods. Natto is a Japanese food made by fermentation of cooked soybeans with *B. subtilis* var. *natto*. Natto carries about 10^8 viable spores per gram of the product (355) and its consumption

has been associated with stimulation of the immune system (364). Nattokinase produced at higher levels by *B. subtilis* var. *natto* has been shown to induce immune stimulation and produce vitamin K2 which can exert anti-cancer properties (365-367). Co-culturing *B. subtilis natto* with *Lactobacillus in vitro* has been shown to improve the viability of *Lactobacillu*s through production of catalase and subtilisin (364). This synergism could be useful in designing functional foods carrying both *Bacillus* and *Lactobacillus* probiotics cells to influence the viability of *Lactobacillus*, in addition to conferring their collective beneficial effects.

Fermented soybean foods based on *Bacillus spp*. have a long history and are consumed in Korea, Japan, China, Southeast Asia and some African countries (355, 368-370). Certain *Bacillus* strains have been credited to improve biological function and the textural and sensory attributes of fermented food products when used as starter culture (371-373). The antimicrobial substances produced by *Bacillus* species inhibit food pathogens (369, 373, 374) and could therefore potentially play a vital role as protective starter cultures against pathogenic microorganisms contaminating foods. This function has been previously designated for bacteriocin producing LAB (375). In addition, *Bacillus* species isolated from various traditional food products have been shown to have probiotic efficacy that presents the means to develop functional foods with health benefits. A fermented soymilk, employing *B. subtilis* as the starter culture and purple sweet potato extract as a fermentable substrate, exhibited high antioxidant activity *in-vitro* (376). Also, *cheonggukjang*, a Korean soybean paste produced with the co-inoculation of *B. subtilis* W42 and *B. amyloliquefaciens* MJ1-4, exhibited high antioxidant, anti-fungal and fibrinolytic activity (377). *Cheonggukjang* fermented with *B. licheniformis*-67 suppressed obesity related parameters in high fat diet induced obese mice (378). Surfactin produced by *B. subtilis* CSY191 during fermentation of *doenjang* (a Korean fermented soybean paste) was shown to inhibit the growth of human breast cancer cells *in-vitro* (379). More in-depth studies exploring the probiotic potential of *Bacillus*-based foods will facilitate the awareness of spore-based probiotics among consumers.

2.3.2.3 Potential probiotic mechanisms of Bacillus in IBD

In contrast to that of conventional lactic acid bacteria (LAB), the mechanisms potentially responsible for the beneficial attributes of *Bacillus* species remained relatively unexplained until fairly recently. Similarly to conventional LAB probiotics, *Bacillus*

probiotics are now known to also mediate benefits to the host via immunomodulation and pathogen exclusion (via antimicrobial production and competition for receptors and nutrients). Oral administration of *Bacillus* spores has also proved effective in modulating immune response and balancing gut microflora in animal and human studies (Table 2.3 and 2.4).

Table 2.3. Experimental studies demonstrating the immune response elicited by *Bacillus* spore application

Bacillus species/strain	Dose	Experimental model	Immune response	Reference
***B. subtilis* A102**	7×10^{11}/g spores	*In-vivo*: ddY mice	• Increased macrophage and NK* cell activities via induction of interferons in mice	(380)
***B. subtilis* PY79**	1.67×10^{11} spores in 0.15 mL volume	*In-vivo*: Balb/C mice	• Increased systemic spore coat-specific IgG levels	(381)
***B. subtilis* PY79**	1×10^{9} spores in 0.15 mL volume	*In-vivo*: C57BL/6 mice	• Increased anti-spore specific IgG titres, • Increased anti-spore faecal sIgA • Increased production of IFN-γ & TNF-α in GALT* and secondary lymphoid organs	(382)
***B. subtilis* (HU58 and HU68), *Bacillus licheniformis* (HU14 and HU53) and *Bacillus flexus* (HU37)**	1×10^{9} spores in 0.2 mL volume	*In-vivo*: Balb/c mice	• Stimulated cell proliferation in germinal centres of Peyer's patches • Increased stimulation of antigen-presenting cells and T lymphocytes	(383)
B. subtilis PB6	1.5×10^{7} and 1.5×10^{8} CFU/mL	*In-vivo*: TNBS-induced colitic Wistar albino rats	• Reduced plasma pro-inflammatory cytokine levels (TNF-α, IL-1β, IL-6, IFN-γ) • Increased plasma anti-inflammatory cytokine levels (IL-10 and TGF-β)	(384)
B. subtilis natto	1×10^{10} CFU in 10 mL volume	*In-vivo*: preweaning calves	• Increased serum IgG and IFN-γ levels in calves	(385)
***B. coagulans* GBI-30, 6086**	2×10^{9} CFU spores	*In-vivo*: Clostridium difficile-induced C57BL/6 colitic mice	• Suppressed activation of NF-ƙB and colonic MIP*-2 content	(386)

***B. subtilis* PB6 (Anaban™)**	1×10^9 CFU spores	*In-vitro*: human PBMCs *In-vivo*: TNBS-induced colitis mice	• Increased IL-10 secretion and decreased TNF-α, IFN-γ and IL-12 in human PBMCs • Decreased systemic IL-6 and myeloperoxidase activity	(387)
***B. subtilis* PY79 (heat-killed) as adjuvant**	1×10^9 CFU spores	*In-vivo*: BALB/c mice challenged with H5N2 influenza virus	• Increased levels of both systemic IgG and mucosal sIgA specific to the H5N1 virion	(388)
***B. subtilis* (natto) B4**	1×10^8 spores/mL medium	*In-vitro*: Murine macrophage, RAW 264.7 cells	• Increased concentrations of TNF-α, IFN- γ, IL-1β, IL-6, IL-10 and MIP-2	(389)
***B. subtilis* and *Enterococcus faecium* (Medilac-S)**	250 mg capsule contains 5×10^7 *B. subtilis* CFU and 4.5×10^8 *E. faecium* CFU	*In-vivo*: TNBS-induced colitic Sprague-Dawley rats	• Reduced percentages of Th1 and Th2 cells but increased the percentage of Treg cells • Reduced expressions of TLR2, TLR4, and TLR9	(390)
***B. subtilis* MBTU PBBMI**	1×10^8 CFU spores	*In-vivo*: Balb/c mice	• Increased serum Ig A and Ig G	(391)
B. subtilis	1×10^8 CFU spores with or without 5-amino salicylic (ASA) acid	In-vivo: DSS-induced colitic Balb/c mice	• *B. subtilis* alone and *B. subtilis* + 5ASA both: ◦ Improved disease activity score and prevented histological damage ◦ Increased expression of TJ proteins (claudin-1, occludin, JAM-A and ZO-1) ◦ Reduced expressions of IL-6, IL-17, IL-23, and TNF-α) • More significant improvement in all above parameters with *B. subtilis* + 5ASA relative to *B. subtilis* alone	(392)
***B. subtilis* R179**	1×10^9 CFU spores (high dose) Or 1×10^8 CFU spores (low dose)	*In-vivo*: DSS-induced C57 colitic mice	• Reduced plasma levels of IL-12, IL-17 and IL-23 with high dose • Increased plasma level of IL-10 with high dose	(393)
***B. coagulans* [Laktovit Forte (LAF)]**	LAF constituents-120×10^6 *B. coagulans* spores,	*In-vivo:* Streptomycin-induced diarrhoea and cyclophospha	• Reduced diarrhoea and body weight loss • Normalised the numbers of splenic lymphocytes, macrophages and T-	(47)

	0.0015g folic acid and 15 g cyanocobalamine at as dose of 46 mg/kg	mide-induced immunosuppression in mice	lymphocytes	
B. coagulans and B. subtilis (natto)	spores used at multiplicity of infection of 1000 in fresh medium	*In-vitro*: HT-29 cells with or without LPS at pre- and post-treatment conditions	• Under pre-treatment conditions, *B. coagulans* compared to *B. subtilis* reduced mRNA expression and secretion of IL-8 • Under post-treatment condition only *B. subtilis* suppressed IL-8 secretion but could not sustain inhibition beyond 3 hours	(394)

∗ = NK- Natural killer, GALT- gut-associated lymphoid tissue, MIP- macrophage inflammatory protein

Immune modulation is one of the vital probiotic attributes that reflects its probiotic effectiveness. There are efforts by some researchers to delineate whether the intact spores can elicit immune response or if their germination into vegetative cells (VCs) is necessary for the probiotic effect. The metabolically inactive nature of spore raises scepticism on their capacity to induce immune response. *Bacillus* spores however may elicit better immune response in GIT compared to their VCs (382). Multiple studies have ascertained the ability of ingested spores to germinate in the murine GIT (382, 395, 396). Nonetheless, the immune response that followed was not confirmed to be triggered solely by the germinated VCs and was partly hypothesized to be mediated by the ability of intact spores being able to trigger immune response by themselves (395). Anti-spore specific serum IgG and faecal secretory IgA titres showed significant immune response following immunization of mice with *B. subtilis* PY79 spores but not with VCs dosing, compared to a naive control group (382). The research supported the conjecture that *Bacillus* spores are immunogenic and can generate specific local and systemic immune responses and hence cannot be considered simply as nutrient food. Ciprandi et al. (397) however, had previously reported the inability of *Bacillus subtilis* spores to influence the immune response, while its vegetative forms enhanced mitogenic-induced T cell proliferation in their study. Different doses and/or strains of *B. subtilis* used in different studies could have contributed to such discrepancies.

Spinosa et al. (398) concluded that the claimed probiotic effect should be attributed to spores or alternatively, to vegetative growth outside the intestine. No detectable levels of VCs of *B. subtilis* in intestinal samples of mice were found while, both VCs and spores were detected in lymph nodes and spleen. Later, in 2008 Huang et al. (383), showed evidence of

cell proliferation in germinal centres of Peyer's patches (PP) following oral administration of *Bacillus* spores. Stimulation of antigen presenting cells and T lymphocytes was also reported to be markedly enhanced. In the same study, *B. subtilis* spores, as autoclaved and germination defective forms, failed to stimulate expression of Toll-like receptor (TLR) genes for TLR2 and TLR4 compared to VCs and intact spores. Hence, they, concluded that spores must interact with another TLR, and by this mechanism help activate innate immunity. Leser et al. (399) concluded that a substantial level of growing VCs in GIT is not a pre-requisite for mode of action of *Bacillus* probiotics. In their experiment 70-90% of the dietary-supplemented *Bacillus* spores germinated in the proximal part of pig GIT, but only limited outgrowth of the VC population was noted. To get a clear understanding of this issue, stringent investigation is needed that compares the immune efficacy between heat-killed spores, intact spores and the vegetative forms for a specific *Bacillus* species/strains.

Nevertheless, substantial numbers of animal studies have demonstrated the ability of orally administered spores to stimulate immune system and exert beneficial effects. Some of the important studies affirming the immunomodulatory efficacy of oral administration of spores have been highlighted in Table 2.3. Following oral inoculation, a small proportion of *B. subtilis* spores have been shown to penetrate Peyer's patches and interact with gut-associated lymphoid tissue (GALT), and to accumulate and germinate in macrophages (381, 382, 395). The small size of spores probably mediates their uptake by M-cells that are localized in the mucosal epithelium of the intestine, and are then further disseminated to the Peyer's patches where they interact with dendritic cells (DCs), macrophages or B cells before their transportation to efferent lymph nodes (381). In another study (384), oral administration of *B. subtilis* PB6 to TNBS-induced colitic rats successfully lowered the plasma pro-inflammatory cytokines (TNF-α, IL-1β, Il-6 and IFN-γ), while, increasing anti-inflammatory cytokines (IL-10 and TGF-β). Similar excellent immunomodulatory and anti-inflammatory effects were confirmed for *B. subtilis* R179 strain in DSS-induced colitic mice while, also reducing mucosal colonic damage and inducing microbial modulations (393).

Spores can therefore directly interact with the immune system to prevent activation of inflammatory mediators (384, 387, 392). A recent *in-vitro* study by Azimirad et al. (394), indicated that the time of spore probiotic treatment could substantially influence its immunomodulatory capacity. In their study, differential immune regulating effects on secretion and mRNA expression of IL-8 with a *B. subtilis* and *B. coagulans* spores in LPS-

induced HT-29 cells were confirmed. *B. coagulans* spores, compared with *B. subtilis* spores under pre-treatment conditions in LPS-induced HT-29 cells, significantly reduced the secretion and mRNA expression of pro-inflammatory IL-8. While, under post treatment conditions, only *B. subtilis*, but not *B. coagulans*, spores suppressed IL-8 secretion although they could not sustain IL-8 inhibition beyond 3 hours. The observations of this study suggested that the immunomodulatory effect may be species/strain specific and time of probiotic treatment to achieve the intended benefits by managing inflammation is a vital factor. It can thus be concluded that application of *B. coagulans* spores before or during the onset of inflammation, will be pragmatic in mitigating an altered immune response in IBD to achieve optimum benefits.

Apart from immunomodulatory capacity, the ability of PB to modulate dysbiosis is an essential attribute for its application in IBD. The antimicrobials produced by probiotics are one of the prime mechanisms that function by inhibiting pathogens in the GIT to create a healthy microbial balance. In this context, *Bacillus* species produce a large number of antimicrobials, including bacteriocins and bacteriocins-like inhibitory substances (e.g. Subtilin and Coagulin) as well as antibiotics (e.g. Surfactin, Iturins A, C. D. E, and Bacilysin) (359, 400). *B. clausii* strains in Enterogermina® have been demonstrated to produce antimicrobials with activity against Gram-positive bacteria (401). *B. coagulans* produces coagulin, a heat-stable, protease-sensitive bacteriocins-like substance with activity against Gram-positive bacteria (402). Lactosporin is an antimicrobial produced by *B. coagulans* ATCC 7050 that has been demonstrated as effective and safe with potential application for the control of bacterial vaginosis (403). Furthermore, *B. coagulans* is capable and industrially used as an efficient producer of lactic acid (404) that is known to inhibit pathogenic growth. The anti-cancer property of surfactin from *Bacillus* species have been affirmed to kill human breast cancer MCF-7 cells through induction of apoptosis (379, 405). While, secretion of these anti-microbial compounds would require the *Bacillus* to be in their vegetative state, the spore administration has also been reported to be able to induce microbial changes in the gut.

A single oral inoculum of 1×10^9 *Bacillus subtilis* spores, given 24 h prior to chickens being challenged with *Salmonella enterica* Serotype Enteritidis and *Clostridium perfringens*, was reported to be sufficient to suppress colonisation and persistence of both pathogens (406). Another study demonstrated the efficacy of oral administration of *B. subtilis* var. *natto* spores in influencing faecal microflora (especially *Bacteroides* and *Lactobacillus* species) in mice depending on the diet (367). In this study, numbers of *Lactobacillus* spp. declined when mice

were fed with an egg white diet but, stabilized when diet was supplemented with *B. subtilis* var. *natto* spores. However, in the case of a casein diet supplemented with spores, the numbers of *Lactobacillus* spp. remained unchanged, while, the numbers of *Bacteroidaceae* increased. Administration of intact spores of *B. subtilis natto* in mice in their study was found to increase faecal *Bacteroidaceae* and *Lactobacillus* counts unlike autoclaved spores. *B. Subtilis* spores have been successfully demonstrated to suppress enteropathogenic infection of *Citrobacter rodentium* in a mouse model of traveller's diarrhoea (407). Consumption of *B. coagulans* and subsequent use of prebiotics was demonstrated *in vitro* by Nyangale et al. (408) in elevating populations of beneficial genres of bacteria (*Faecalibacterium prausnitzii*, *Clostridium lituseburense*) as well as SCFA production in faecal microbiota of elderly volunteers. In a following placebo-controlled study by the same group of workers, consumption of *B. coagulans* spores (GBI-30, 6086) increased numbers of beneficial *F. prausnitzii*, in humans (409). Oral administration of skim milk supplemented with *B. coagulans* B37 and *B. pumilus* B9 spores in rats decreased faecal coliform counts with concurrent increase in *Lactobacillus* count in treatment group (410). Feeding of *B. coagulans lilac-01*, along with soya pulp, to cholic acid fed rats, suppressed the production of secondary bile acid, improved gut permeability and lowered the bactericidal effect of bile acid which in turn supported the growth of beneficial intestinal microbiota (411). In a recent study, *B. subtilis* administration in mice ameliorated dextran sulphate sodium (DSS)-induced dysbiosis and gut inflammation by balancing beneficial and harmful bacteria (393). *B. subtilis*-treated colitis mice group showed specific reduction of *Acinetobacter* spp., *Ruminococcus* spp., *Clostridium* spp. and *Veillonell*a spp. with increase in members of *Bifidobacterium* spp. *Lactobacillus* spp. and *Butyricicoccus* spp. In addition to a beneficial effect on the gut microbiota and an immune-regulating effect in DSS-induced mice, significant ability of these spores in increasing total SCFA levels and preventing the damage to intestinal barrier function was also recorded. Increased expression of mucin, recovery of intestinal permeability and increased expression of TJ proteins were shown to contribute to the recovery of DSS-induced injury by the administration of *B. subtilis* spores (393). The application of *B. clausii* spores in the treatment and prevention of gut barrier impairment has also been largely supported in the last years (412).

The excellent ability of *Bacillus* spores to induce immunomodulation, coupled with its efficacy to balance gut flora and restore gut barrier, has heralded its application as probiotic in human application in managing GIT inflammatory conditions. Some of the prominent clinical trials demonstrating the efficacy of *Bacillus* probiotics in human health including

gastrointestinal conditions are listed in Table 2.4. Oral administration of *Bacillus* spores has been affirmed to reduce abdominal pain and bloating in patients with irritable bowel syndrome (IBS) and improve the quality of life (48, 413, 414). *Bacillus* spores, as an adjuvant with antibiotics, has proved effective in reducing the incidence of antibiotic-associated diarrhoea and adverse effects related to antibiotics (415, 416). Apart from showing benefits in gut-related conditions, *Bacillus* spores have been determined to be effective in alleviating other inflammatory conditions in clinical studies of rheumatoid arthritis (417), respiratory infections (46) and generalized gingivitis (418). In a recent diet-controlled study, *B. coagulans* in combination with casein protein significantly enhanced post-exercise recovery while decreasing muscle soreness in recreationally trained males (419). In addition to conferring direct benefits over host health, natural food products involving *Bacillus* strains are now being explored in the management of systemic clinical syndromes including metabolic disorders (378, 420, 421). Therefore, the excellent ability of *Bacillus* spores to modulate gut health makes it an attractive choice as functional probiotic ingredient in pharmaceuticals and foods targeted for improved gut health and resolving gut inflammation in IBD.

Table 2.4. Beneficial effects of probiotic *Bacillus* spores in humans

***Bacillus* strain and dose**	**Type of subjects**	**Participants (treatment/control) and Duration of Study**	**Effects/outcome**	**Reference**
***B. subtilis* 3 and *B. licheniformis* 31 or *B. subtilis* 3 alone –** 2×10^9 CFU/vial in combination with antibiotic	Patients suffering from antibiotic-associated diarrhoea (AAD)	*n*= 271 adults (91/90/90), 7 days	• Probiotic mix and *B. subtilis* 3 alone decreased the incidence of AAD and adverse effects (nausea, bloating, vomiting and abdominal pain) related to antibiotics • No Significant differences found in efficacy of strains	(415)
***B. subtilis* CU1** – 2×10^9 Spores/day	Healthy elderly subjects with history of common infectious diseases (influenza)	*n*= 100 elderly (50/50), 4 months	• Increased faecal and salivary secretory IgA and IFN-γ concentrations • Decreased frequency of respiratory infections	(46)

***B. coagulans* GBI-30, 6086 –** 1×10^9 CFU/capsule/day	Healthy elderly subjects	*n*= 39 elderly, 28 days crossover with 21 days washout period	• Increased populations of *F. prausnitzii* during probiotic consumption • Peripheral blood mononuclear cells (PBMCs) showed increase in IL-10 after stimulation with LPS	(409)
***B. coagulans* GBI-30, 6086 –** 8×10^6 CFU/day	Patients with diarrhea-prominent IBS	*n*= 44 adults (22/22), 8 weeks	• Reduced abdominal pain and bloating significantly	(414)
***B. coagulans* GBI-30, 6086 –** 2×10^9 CFU/caplet/day as an adjuvant with anti-arthritic medications	Patients with rheumatoid arthritis	*n*= 45 adults (23/22), 60 days	• Improvement in the pain assessment score and pain scale from baseline • Reduction in CRP	(417)
***B. coagulans* MTCC 5856 –** 2×10^9 CFU/tablet/day	Diarrhoea predominant IBS patients	*n*=36 adults (18/18), 90 days	• Decreased clinical symptoms like bloating, vomiting, diarrhoea, abdominal pain and stool frequency • Reduced disease severity and improved quality of life	(48)
***B. clausii* (*Enterogermina*) –** 2×10^9 spores/vial/day thrice as adjuvant with anti-*H. pylori* medication	*H. pylori*-positive patients	*n*= 120 adults, 14 days	• Reduced incidences of nausea, diarrhoea and epigastric pain • Lowered the intensity of the nausea and diarrhoea related to anti-*H. pylori* antibiotic therapy	(416)
***B. subtilis*, *B. megaterium* and *B. pumilus* –** 5×10^7 CFU contained in toothpaste and mouth rinse	Healthy patients with generalized gingivitis	*n*= 40 adults (20/20), 8 weeks	• Reduced plaque and gingivitis indices	(418)
***B. coagulans* GBI-30, 6086 –** 2×10^9 CFU/capsule/day	Patients with self-reported post-meal intestinal gas-related symptoms (including abdominal pain, cramps, distended feeling/bloating, and	*n*= 61 adults (30/31), 4 weeks	• Improved quality of life • Reduced gastrointestinal symptoms like abdominal pain and abdominal distension sub scores	(422)

	flatulence)			
***B. coagulans* Unique IS-2 –** 2×10^9 CFU/capsule/day twice	Patients with acute diarrhoea	*n*= 28 adults, 10 days	• Decreased mean values for duration of diarrhoea • Reduced frequency of defecation • Lowered abdominal pain • Improved stool consistency	(49)
***B. coagulans Unique IS-2* –** 2×10^9 CFU/chewable tablet/day twice	Children with diagnosed IBS	*n*= 141 children (72/69), 8 weeks with washout period of 2 weeks	• Reduced pain intensity • Improved stool consistency • Reduced abdominal discomfort, bloating, staining, urgency, incomplete evacuation and passage of gas	(423)
***B. coagulans* (Colinox®) –** 1.5×10^9 CFU/g composition, 3 times/day	Patients with IBS	*n*= 52 adults (26/26), 4 weeks	• Reduced bloating, discomfort and pain	(413)
***B. clausii* (*Enterogermina*) –** 2.4×10^9 spores/ day	Preterm neonates	*n*= 244 babies aged <36 weeks (123/121), 6 weeks	• No significant difference in the incidence of late-onset sepsis (LOS) • Full feeds achieved significantly faster in probiotic group	(424)
***B. coagulans* GBI-30, 6086 and casein -**1 $\times 10^9$ CFU spores or – casein (20 g)	Recreationally-trained males	*n* = 29 adults, 7 weeks with 1 week of washout period	• Spores and casein combination increased post-exercise perceived recovery and decreased muscle soreness • Spores alone showed a trend towards reduced muscle damage • Strenuous exercise reduced athletic performance with spores alone, while those on a combination, maintained performance	(419)

2.3.2.4 Bacillus coagulans spores as potential probiotic ingredient in IBD

Many *Bacillus* species have been examined for their probiotic efficacy including *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus clausii*, *Bacillus cereus*, and *Bacillus licheniformis* (359). *Bacillus* probiotics are commercially available for use in humans as dietary supplements, in animals as growth promoters and in aquaculture for promoting growth and disease-resistance (355). So far commercial products of *Bacillus* composing functional foods are not popular in the nutraceutical market because the debate over probiotic versus pathogen tag of *Bacillus* species is still persisting. Hence, it is important to accurately examine the phenotypic and genotypic characteristics of selective *Bacillus* species and their substantiation with those having generally regarded as safe (GRAS) status, to reach a consensus over the same (425). Nevertheless, apart from the application of probiotic *Bacillus* spores in dietary supplements, they are also being incorporated and explored into variety of food matrices to provide added health benefits beyond basic nutrition (43, 377, 378, 426).

Bacillus coagulans (earlier known as *Lactobacillus sporogenes*) spores are one of the most promising spore probiotic candidates and now being incorporated as probiotics ingredient owing to the excellent stability during processing and storage of the products and gastric transit (47, 64). The exceptional stability of *B. coagulans* spores at manufacturing and storage temperatures as well as its ability to survive the gastric acidity validates the incorporation of *Bacillus* spores as probiotic ingredient in foods and pharmaceuticals (43, 44). In addition to their distinguished stability, ease of incorporation in to food as well as approved GRAS status of certain *B. coagulans* spores has encouraged its utilization in the number of commercial food products (64). Moreover, germination of these spores does not occur in many foods and hence the product quality is not affected because of their inactive metabolism (427). The capacity of *B. coagulans* to withstand high temperature processes, and maintain viability and stability relative to commonly applied probiotic strains, is being explored to formulate functional foods that require baking and boiling.

Some commercial probiotic formulations containing *B. coagulans* spores are currently available as ingredients including *B. coagulans* MTCC 5856 (marketed as LactoSpore®) and *B. coagulans* GBI-30, 6086 (marketed as GanedenBC30) and are characterized by the ability to survive manufacturing processing (including mild heat-treatments) and long shelf-life, yet

retaining its probiotic properties (43, 426). Fares et al. (426) formulated pasta incorporating *B. coagulans* GBI-30 spores utilising wheat flour rich in polyphenols. The probiotic strain in their study, maintained viability during the pasta-making and cooking processes (~ 9.0 Log CFU/ 100g). In another study (43), *B. coagulans* MTCC 5856 spores were found to be stable in variety of food products during processing and storage with different nutritional profiles. The PB was showcased to be stable during baking and storage at frozen conditions of banana muffins (92% viability) and waffles (86% viability) for up to 12 months. Moreover, over 95% spore viability was recorded in chocolate fudge frosting, hot fudge toppings, peanut butter, strawberry preserve and vegetable oil at room temperature up to 12 months. An 87% viability was exhibited by *B. coagulans* spores after brewing with coffee at 90 °C for 2 minutes and retained 66% viability even after maintaining temperature at 77 °C for 4.0 h (43). Furthermore, probiotic spores were found to be stable in apple juice up to 6 months at refrigerated condition and concentrated glucose syrup at 4±3 and 25 ± 2 °C for up to 24 months. The stability of *B. coagulans* spores, in range of food products with different nutritional profiles and variation in the proximate parameters such as protein, fat, carbohydrate and moisture, validate its suitability to be incorporated as stable probiotic in variety of functional food matrices. Moreover, its stability in food during room temperature storage can be employed to incorporate these probiotic agents in novel foods that are shelf-stable without refrigeration, which is unlikely to be achieved in the case of commonly applied LAB. In light of the above reports, it can be stated that *B. coagulans* spores are an excellent choice for stable application in functional foods as probiotic ingredients.

Despite a number of drugs containing *B. coagulans* having entered the global pharmaceutical market, and already proven their clinical efficacy (428), the pharmacological effects and the underlying mechanisms of action of this spore probiotic still remains poorly understood. While, excellent immunomodulatory capacity of *B. coagulans* have been demonstrated in most studies (47, 386, 409, 429, 430), a few studies have also reported its antiviral activity (431, 432) and capacity to modulate gut microbiota (433). *B. coagulans* spores have been shown to be of benefit in number of GIT conditions including antibiotic-associated diarrhoea (434, 435), IBS (48, 413, 414, 436) and flatulence (422) in humans. *B. coagulans* has been shown, not only to improve the indices (386), but also reduce the recurrence of *C. difficile*-induced colitis in mice (433). The study confirmed beneficial effects of *B. coagulans* spores in improving stool consistency and attenuating colonic histological and biochemical indices in mice. *B. coagulans* has also been shown to be effective in treatments of viral conditions (432), dental caries (437) and vaginitis (438). However, their

mechanisms of functional efficacy are still not completely understood, and efforts are underway to evaluate their mechanistic action. Although *B. coagulans* have been shown to be effective in ameliorating experimental colitis in mice and reducing diarrhoea and related GIT conditions in humans, its efficacy in human IBD patients has not been explored. This may be due to lack of understanding of its mechanistic action. More in-depth *in-vivo* and *in-vitro* studies focussing on delineating the mechanistic functionality of *B. coagulans* spores are required to encourage increased therapeutic application in human IBD and in functional foods aimed at maintaining gut health.

2.3.3 Synbiotics in IBD

2.3.3.1 Synergistic and complementary synbiotics

The combination of prebiotic and prebiotic is termed as synbiotic (24) and the encompassing synergy between the two components is thought to augment the beneficial effects on the host. The synbiotic concept was first introduced in 1995 and two types of synbiotic approaches exist (55):

- Complementary synbiotic, whereby the probiotic is chosen based on the specific desired effects on the host, and the prebiotic is independently chosen to selectively increase the level of the beneficial microbiota. The prebiotic may promote the growth and activity of the probiotic, but only indirectly as a part of its target range.
- Synergetic synbiotic, whereby the probiotic is chosen based on specific beneficial effects on the hosts, but the prebiotic is chosen to specifically stimulate the growth and activity of the selected probiotic. Here, the prebiotic is selected to have higher affinity for the probiotic and is chosen to improve its survival and growth in the host. It may also increase the level of beneficial host gastrointestinal microbiota, but the primary target is the ingested probiotic.

Kolida and Gibson (55) explained that, in a complementary approach, each component is administered in a such a dose as to elicit a desirable effect via the vehicle of the administration which usually requires a relatively high prebiotic dose to mediate an effect on the gut microbiota. In contrast, with synergistic approach, synbiotic is perceived as a single product, whereby the primary role of the prebiotic is to enable the survival and activity of the probiotic. The necessary dose of prebiotic may be limited to this effect alone, and hence, a

smaller dose of the probiotic is required (55). Moreover, while both approaches may directly or indirectly comply with the synbiotic definition, it is the synergistic approach that has most pragmatic implications on the host health. While, the current synergistic synbiotic approach limits the role of prebiotic in influencing the growth and activity of the probiotic, the direct prebiotic beneficial effects on the gut microbiota, as well as on the colonic barrier integrity and immune regulation, also need to be accounted for. In the synergistic approach, the potential to elicit the combined beneficial effects of probiotic or prebiotic owing to synergism is greater. This includes increased SCFA production and thus results in mediating potentiated gut health benefits to the host.

2.3.3.2 Synergistic synbiotic – a two-point approach for resolving the inflammatory loop in IBD

Synergistic synbiotic seems to be pragmatic two-point approach in mitigating inflammatory loop in IBD. Each bioactive component could function independently (e.g. direct immune-regulating effects, effects on colonic barrier integrity or influence on the gut microbiota profile) while also mediating the synergistic benefits by interaction of probiotic with prebiotic to stimulate increased SCFA production via fermentation thus, targeting the overall inflammatory circuit of IBD. For synbiotics, a small number of preliminary *in vivo* studies have been performed relative to that of probiotic and prebiotic and the focus has been almost exclusively on disease management. Experimental studies on DSS-induced colitis rat model testing the pre-treatment effect of administration of *Bifidobacterium infantis* DSM 15158 or *B. infantis* DSM 15159, alone or in combination with Synergy1 (Oligofructose and inulin), observed marked attenuation in disease markers (i.e. bacterial translocation, SCFA, cytokine production, myeloperoxidase and malonaldehyde) (439). While, all treatments induced a significant improvement in the DAI, an additive effect was noted when Synergy1 with *B. infantis* DSM 15159 was used. It was noted to mediate an increase in succinate production and subsequently suppress neutrophil infiltration. This was a well-defined study comparing the effect of each of the synbiotic constituents alone and in combination and investigating the effect of strain specifically against the disease. In another study, *Lactobacillus paracasei*, combined with FOS and arabinogalactan, also showed curative effects on DSS–mice model of colitis (440). *L. plantarum* 299 with oat fibre however had no effect on established TNBS-induced colitis in rats (441).

There are not many well-designed clinical studies investigating the effect of synbiotics for IBD. In a small randomised, placebo-controlled study (442) with 18 active UC patients, administration of synbiotic Synergy 1 (combination of *B. longum* and with OFI) twice daily for a month, produced statistically significant reduction in β-defensin, TNF-α and IL-1α, more epithelial regeneration of on mucosal biopsies, and a trend towards a reduction in endoscopically visualized levels of inflammation. The study also followed a rational procedure for probiotic selection that would complement the selected prebiotic as well as target the disease. A selection of 19 *Bifidobacterium* isolates were screened for their suitability as probiotic in terms of aerotolerance, acid tolerance, bile-salt tolerance, adhesion to epithelial cells and their ability to survive freeze drying and long-term storage. The ability to metabolise FOS as an energy source, as well as the ability of the strains to reduce production of pro-inflammatory cytokines in the HT-29 epithelial cells, was also determined. In another large open label study with 120 active UC patients (86), significant improvement in their health-related quality of life after receiving synbiotic combination after 4 weeks was reported, but a similar effect was not observed for patients receiving either probiotic *B. longum* or prebiotic psyllium. Moreover, only synbiotic patients were noted to have reduced CRP levels. In a separate study, administration of synbiotic combination of *B. breve* and GOS administration in 41 UC patients resulted in improvement in endoscopically defined levels of inflammation in the synbiotic group when examined after 1 year of follow-up. The synbiotics significantly reduced the faecal counts of *Bacteroidaceae* and faecal pH (443). A multicentre, randomized, placebo-controlled study (444) investigating the efficacy of synbiotic 2000 (10^{10} CFU of each *Pediacoccus pentoseceus, Leuconostoc mesenteroides, L. casei* spp. *paracasei* F1977:1, *Lactobacillus plantarum* 2362, and 2.5 g each of β-glucans, resistant starch, inulin, and pectin; Medipharm) in 30 ileal resection CD patients over a period of 24 months failed to note an effect on remission or disease scores compared to placebo.

Research in IBD using synbiotic treatments is therefore still in its infancy. It is evident, however, that when an informed selection of the probiotic and complementary prebiotic that can function synergistically is made, pilot studies have been successful. Although, the considerable efficacy of synbiotic therapy in imparting benefits is appreciated, the challenge however, is to determine the best combination of probiotic and prebiotic in order to achieve maximum benefits. Geier et al. proposed that the first attempt should be focussed on combining probiotics and prebiotics that have demonstrated individual benefits to determine if there are any synergistic effects, followed by a more structured approach that would determine the specific attributes that a prebiotic requires to be beneficial to the

probiotic and select the prebiotic accordingly (445). The selection of prebiotic and probiotic for an effective synbiotic should not only focus on the ability of prebiotic to support the growth and survival of probiotic, but also the ability of a probiotic to efficiently metabolise the selected prebiotic to generate increased SCFA that are known to be beneficial in resolving inflammation in IBD.

2.4 Conclusions

The current IBD paradigm focusses on the tripartite pathophysiological circuit that encompasses three distinct inflammatory features: the altered colonic epithelial integrity, dysregulated immune response and dysbiotic microbiota. The limited efficacy of current therapies focusing primarily on immunosuppression highlights the need for an alternative and relatively pragmatic approach that would target to resolve the overall inflammatory loop of IBD. With the considerable efficacy in targeting these distinct features of the IBD inflammatory circuit, prebiotic DF and probiotic are developing as preventive and corrective treatment therapies for IBD. Synbiotic, which is a two-point approach carrying probiotic and prebiotic components, seems to be a pragmatic approach owing to the potentiated synergetic benefits in mitigating inflammation IBD. To achieve augmented benefits from the synbiotic therapy, the best combination of probiotic and prebiotic is vital. Thus, there exists a possibility to develop synbiotic combinations of compatible probiotics and prebiotics with established health benefits and those that function synergistically to augment the synergistic health outcomes. A careful selection of DF with biochemical complexity and probiotic with marked stability and functionality to generate potent synbiotic preparation is a key to achieve its augmented benefits. On this premise, synergistic combination of *B. coagulans* spore (with its marked immunomodulatory capacity coupled with its ability to metabolise wide variety of plant cell wall material), with prebiotic dietary fibre supplements such as whole-plant prebiotic sugar cane fibre and green banana flour with complex dietary fibre content and other intrinsic bioactive components could be explored to prevent or mitigate the onset of inflammatory circuit in IBD. The information could then be applied to develop novel functional shelf stable synbiotic food products targeted for preventing and treating gut inflammation in humans. Pre-clinical analyses using suitable *in-vitro* and *in-vivo* models to determine the efficacies of the synbiotic ingredients in improving the outcomes of the inflammatory gut conditions will be pragmatic before application in humans.

Chapter 3

Probiotic *Bacillus coagulans* MTCC 5856 spores exhibit excellent *in-vitro* functional efficacy in simulated gastric survival, mucosal adhesion and immunomodulation

3.1 Abstract

Probiotic *Bacillus coagulans* MTCC 5856 spores were evaluated *in-vitro* for their ability to survive simulated digestion, adhesion to colonic cells and immunomodulatory properties. The spores showed significant survival (92 %) during simulated digestion and substantially adhered to the human colonic cells HT-29 (86%) and LS174T (81%) compared with the *L. acidophilus* control. They exerted marked immunomodulatory effects in HT-29 cells, by reducing IL-8 and increasing IL-10 secretion. Moreover, they exhibited pronounced differential immunomodulatory efficacy in response to lipopolysaccharide-induced inflammation under co-treatment (increased IL-10 and reduced IL-8) relative to post-treatment (reduced IL-8 with no IL-10 detection) in HT-29 cells. This observation supports the application of *B. coagulans* spores before or during the onset of inflammation to maximise the probiotic benefits in treating inflammatory bowel conditions. The results provide additional evidence of the probiotic properties of *B. coagulans* spores and support their incorporation into functional foods for improved gut health.

3.2 Introduction

Probiotics are “live microorganisms which, when administered in adequate amounts, confer various health benefits to the host” (29). Many studies have confirmed the therapeutic efficacy of probiotic bacteria when applied to the treatment of several gastrointestinal diseases including diarrhoea, IBD and irritable bowel syndrome (30). In addition to their therapeutic application as pharmaceuticals, probiotics are being incorporated into a variety of food products. Currently, refrigerated dairy products that carry probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* strains are the major delivery forms for probiotic food. The colder storage temperatures and rich growth media assist retention of viability for these conventionally used probiotic strains (353). However, certain strains of *Lactobacillus* and *Bifidobacterium* are reported to be sensitive to gastric transit (38). Despite the commercial success of dairy probiotics, consumers have a genuine interest in non-dairy products (446)

due to lactose intolerance, cholesterol content and the trend towards vegetarianism. Therefore, alternative and convenient probiotic food formats that can be stored without refrigeration, such as breakfast cereals, pasta, cookies and snack bars are being explored (447).

In this context, the ability of *Bacillus* species to form spores has been explored to develop shelf-stable food products. Spores confers higher resistance to technological stresses encountered during industrial production and storage processes and greater protection against hostile gastric and intestinal conditions (pH, bile and digestive enzyme) (63, 65, 66). These characteristics are not displayed by all species of *Lactobacillus* (448). They enable more sustained viability and thus support their incorporation into wider and novel delivery formats.

B. coagulans is a spore-forming, Gram-positive, facultative anaerobic, L (+) lactic acid producing organism. Products supplemented with *B. coagulans* can be stored at room temperature without any deleterious effect on their viability (43). It is one of the most promising spore-forming probiotics, is currently available as a dietary supplement worldwide (449), and has been reported to support healthy digestive (48, 422) and immune functions (432, 450). However, the behaviour and mechanisms of immunomodulatory and anti-inflammatory influence of *B. coagulans* within the gastrointestinal tract is unclear. The functional attributes that reflect probiotic efficacy include viability and persistence in the gastrointestinal tract, adhesion to intestinal cells, antagonism against bacterial infection, and immunomodulation. Few studies have been performed to affirm the immunomodulatory ability of probiotic bacteria belonging to *Bacillus* species, particularly in their spore form. Such information is necessary for appreciating the probiotic effect of *B. coagulans* spores for its application in human health. This study aimed to investigate the gastric stability, adhesion capacity, cytotoxic effect and immune modulatory attributes of *B. coagulans* spores *in-vitro* not previously explored.

3.3 Materials and methods

3.3.1 Bacterial strains and media

LactoSpore® containing probiotic strain *Bacillus coagulans* MTCC 5856 (6×10^9 spores/gm) was supplied by Sabinsa Corporation (Australia) and was produced by Sami Labs Limited (Bangalore, India). LactoSpore® is a commercial proprietary preparation of Sabinsa Corporation, USA which contains spores of *B. coagulans* MTCC 5856 (bearing internal reference number SBC37-01). *Bacillus coagulans* MTCC 5856 has generally regarded as safe (GRAS) status (GRAS Notice No. GRN 000601) approved by the US FDA. *B. coagulans*

MTCC 5856 (*B. coagulans*) spores at a dose of 2 × 10^9 CFU/gm were tested in this study. Viable spore counts in the test sample were determined at the end of adhesion and gastric survival assays following the method of Majeed, et al. (43) with slight modification. Briefly, the test sample was incubated in a water bath for 30 min at 75 °C, followed by immediate cooling to below 45 °C. This suspension was further serially diluted in sterile saline and the viable count was enumerated by spread plating on glucose yeast extract agar (GYEA), following their incubation at 37 °C for 48 to 72 h. GYEA was prepared in-house following the recipe from USPC monograph (451). Each analysis was performed in triplicate and the average mean of spore viable counts are expressed in Log CFU/mL. *Lactobacillus acidophilus* DDS-1 was obtained in freeze-dried, free-flowing lyophilized form from UAS labs, Madison, WI, USA. It was used as the control for *in-vitro* simulated digestion and adhesion assays against *B. coagulans* spores at a dose of 2 × 10^9 CFU/mL. Viable counts of *L. acidophilus* were determined by spread plating on De Man Rogosa (MRS) agar supplemented with 0.05% (w/v) L-cysteine following their incubation at 37 °C for 48 h. The overall experimental design of the *in-vitro* screening of *B. coagulans* spores is illustrated in Figure 3.1.

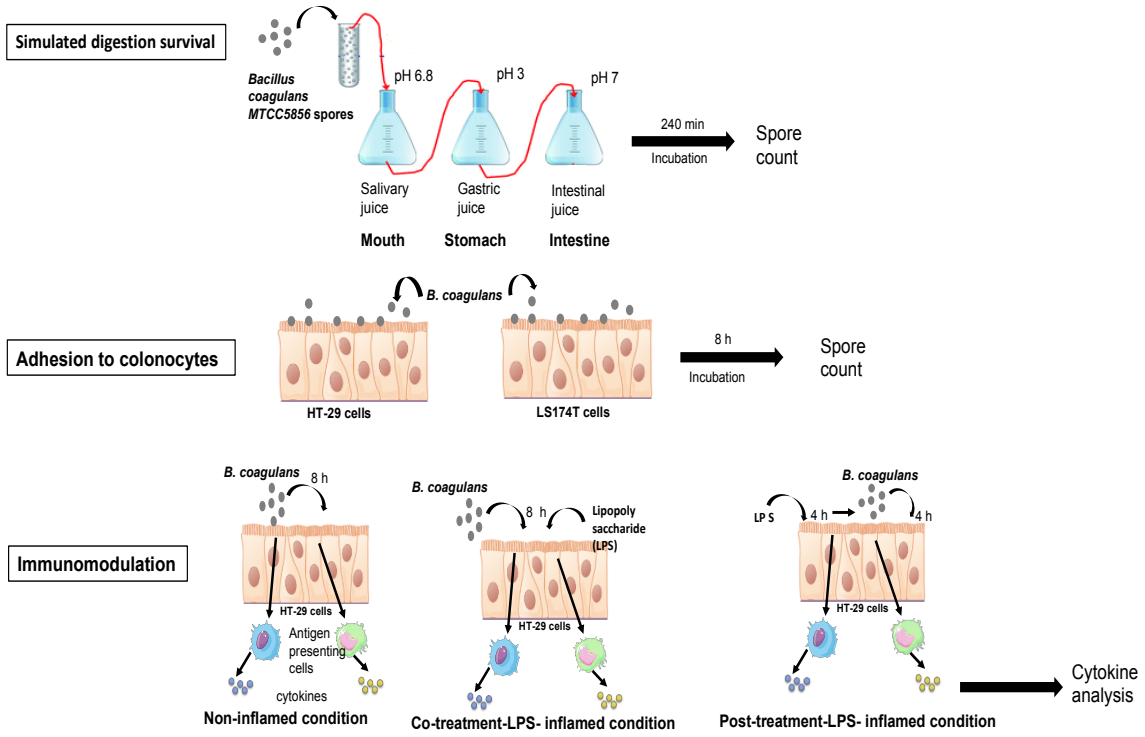


Figure 3.1. The *in-vitro* experimental design for probiotic screening of *B. coagulans* spores. The capacity of *B. coagulans* spores to survive the simulated digestion, adhere to human colonic HT-29 and LS174T cells and modulate immune response in LPS-induced HT-29 cells were evaluated.

3.3.2 Tolerance of B. coagulans spores to the in-vitro simulated digestion

A static *in-vitro* digestion model was designed to assess the survivability of *B. coagulans* spores. The methods of Belguesmia et al. (452) and Chávarri et al. (453) were followed for the preparation of simulated saliva juice and simulated gastric and intestinal juices respectively (Figure 3.1). This model reproduced the temperature, pH, bile salts concentration and enzymes involved in the digestion processes, mimicking the physiological conditions in the gastrointestinal environment. *L. acidophilus* strain was used as control. Bacterial spores and *L. acidophilus* cells were exposed to the gastric stressors, as they would be encountered during gastric transit starting from the mouth, followed by stomach, and then the intestine. One mL of respective bacterial aliquots (at a concentration of 2×10^9 CFU/mL) were separately added to 9 mL of simulated salivary juice (SSJ) [KCL (0.894 g/L), NaH_2PO_4 (0.887 g/L), Na_2SO_4 (0.568 g/L), $NaHCO_3$ (1.680 g/L), CO $(NH_2)_2$ (0.198 g/L)] and adjusted to pH 6.8 ± 0.2. This was then incubated at 37 °C for 5 min to represent buccal conditions. Following the treatment, the *B. coagulans* spores and the *L. acidophilus* cells were pelleted by centrifugation at 3,000 rpm for 5 min to recover spores for the gastric step. After discarding the supernatant, the pellets (containing spores and cells) were resuspended in 9 mL of simulated gastric juice (SGJ), consisting of 9 g/L of sodium chloride containing 0.3% pepsin (Sigma-Aldrich) and adjusted to pH 3.0 ± 0.2. SGJ was then incubated at 37 °C for 2 h. At the end of the incubation step, the SGJ was then neutralised immediately by washing with phosphate buffered saline (PBS) at pH 7. This was then followed by centrifugation at 3,000 rpm for 5 min to recover *B. coagulans* spores and the *L. acidophilus* for the subsequent intestinal step. The pellet was resuspended in 9 mL of simulated intestinal juice (SIJ) which was prepared by dissolving bile salts (0.3% w/v; Sigma-Aldrich) and pancreatin (0.1% w/v; Sigma-Aldrich) in sterile saline solution (0.5% w/v) and adjusting to pH 7.5. SIJ carrying spores/bacteria was then further incubated at 37 °C for 2 h. After each step of the simulated digestion process, samples were collected and evaluated for the spore and *L. acidophilus* cell survival according to the enumeration method described in Section 3.3.1 for the respective bacteria. The analysis was performed in triplicate for three independent experiments. Average mean of *B. coagulans* spore and *L. acidophilus* viable counts are expressed in Log CFU/mL.

3.3.3 Cell lines and culture

Human mucus secreting colonic adenocarcinoma cell lines, HT-29 (ATCC® HTB-38™) and LS174T (ATCC® CL-188™) cells were purchased from American Type Culture Collection (ATCC), Virginia, USA. HT-29 cells were cultured in McCoy's 5a (Modified) Medium (Gibco, Life Technologies, Melbourne, Australia) while, LS174T were cultured in RPMI 1640 Medium (Gibco, Life Technologies) supplemented with 10% foetal bovine serum (Gibco, Life Technologies), L-glutamine (300 mg/L), and 100 U/mL each of penicillin and streptomycin. Cells were cultured in 5% CO_2 at 95% relative humidity and 37 °C. Media was replaced every 2-3 days. When cells reach 80% confluence, the spent medium was completely removed 24 h prior to each experiment and the cells were fed with fresh medium lacking antibiotics to avoid damage to bacterial cells. At confluence, the cells were detached from the flasks using trypsin-EDTA solution and reseeded at a density of approximately 5×10^4 cells/mL in 24-well cell culture plates (Greiner CELLSTAR®, Sigma-Aldrich) for the adhesion, cytotoxicity and cytokine experiments.

3.3.4 Adhesion capacity

B. coagulans spores and *L. acidophilus* cells (control) were suspended in the respective cell culture media devoid of serum and antibiotics at a concentration of 2×10^9 CFU/mL in 24 well plate. Respective bacterial suspensions (1 mL) were then applied separately on confluent monolayers of HT-29 and LS174T cell lines in 24-well plate following the method of Belguesmia et al. (452). After 4 h of incubation at 37 °C with 5% CO_2, monolayers were washed three times with sterile Hank's Balanced Salt Solution (HBSS) to remove non-adherent bacteria. 1 mL saline was then added to each well, the cells were scraped, and the liquid aspirated into a pipette to loosen the cells from the plate surface. These suspensions containing adherent bacteria were then serially diluted and plated onto their respective agars to determine the viable count number as described previously in Section 3.3.1. The experiment was performed in triplicates for three independent experiments and results are expressed as percentage of adhesion.

3.3.5 Cell viability and cytotoxicity assays

The effect of *B. coagulans* spores on the cell viability after 8 hours of incubation was assessed using the level of Lactate Dehydrogenase (LDH) in culture supernatants and the Trypan blue dye exclusion test following the method of Shastri et al. (454). *B. coagulans*

MTCC 5856 spores were suspended in respective cell culture media devoid of serum and antibiotics at a concentration of 2×10^9 CFU/mL. Spore suspensions (1 mL) were then applied to each well on confluent monolayers of HT-29 and LS174T cells in each in 24-well plate. The plates were then incubated for 8 h at 37 °C with 5% CO_2. HT-29 and LS174T cells untreated with *B. coagulans* spores served as controls. The samples were then collected for two different assays as follows:

LDH assay: At the end of incubation, culture supernatants from both HT-29 and LS174T cells were collected for testing LDH activity. Cytotoxicity of spore treatment was investigated using the LDH *in-vitro* toxicology assay kit (Sigma-Aldrich, NSW, Australia), according to the manufacturer's instructions. Briefly, respective cell culture supernatants were centrifuged at 250 × *g* for 4 min. An aliquot containing 50 µL of cleared supernatant was mixed with 100 µL of a solution containing LDH assay substrate, LDH dye and LDH cofactor and incubated at room temperature for 20 min before the reaction was terminated by the addition of 15 µL of 1 N hydrochloric acid. Absorbance at 490 nm was measured spectrophotometrically using a plate reader (Spectra Max M2 microplate reader, Sunnyvale, CA). Each sample was measured in triplicate.

Trypan blue dye exclusion test: At the end of incubation, the cells were scrapped and aspirated with a pipette to loosen the treated and untreated cells from the plate surface and the cell suspensions were collected. Cell viability was examined using Trypan blue (Sigma) exclusion stain with a Countess™ automated cell counter (Invitrogen™, Thermo Fisher Scientific). This analysis was performed in triplicate for three independent experiments.

3.3.6 Cytokine analysis

The efficacy of *B. coagulans* spores to exert immunomodulatory effects on two cytokines, pro-inflammatory IL-8 and anti-inflammatory IL-10 was determined on HT-29 cells challenged by lipopolysaccharide (LPS) treatment following the previous method (455). HT-29 cells were selected for the cytokine assay as the enterocyte-like HT-29 cells represent a well characterised model to study the enterocyte immune response to bacterial infections (456, 457). *B. coagulans* spores (2×10^9 CFU/mL) were suspended in McCoy's 5a medium devoid of serum and antibiotics. HT-29 confluent monolayers in 24 well plate were subjected to probiotic/LPS treatment under the following conditions: (a) Non-LPS-stimulated cells and treated with *B. coagulans*:1 mL of *B. coagulans* spore suspension (2×10^9 CFU/mL) was

added to the wells with confluent HT-29 cell monolayers and incubated at 37 °C, 5 % CO_2 for 8 h. (b) Co-treatment with *B. coagulans* of LPS-stimulated cells: 1 mL (2×10^9 CFU/mL) of probiotic spore suspension and 100 ng/mL LPS (Lipopolysaccharides from *Escherichia coli* K-235; Sigma Aldrich) were simultaneously added to the cell monolayer and incubated at 37 °C, 5% CO_2 for 8 h, (c) Post-treatment with *B. coagulans* of LPS-stimulated: cells were initially challenged with LPS and incubated for 4 h at 37°C, 5% CO_2. Following this, probiotic spore suspension (2×10^9 CFU/mL) was then applied to the LPS-treated cells and incubated for additional 4 h 37 °C, 5% CO_2. HT-29 cells without probiotic treatment or LPS served as a negative control, while LPS – HT-29 cells incubated with LPS for 8 h served as a positive control. All the supernatants were collected and centrifuged at 1,000 × *g* for 15 min at 4 °C. The supernatant was then collected and used for quantification of IL-8 and IL-10 cytokines using a Bio-Plex Pro™ Human Cytokine Assay (Bio-Rad®, Australia). The cytokine results were read on a Bio-Plex® 200 Systems instrument. The experiments were performed in triplicate and the results are reported as mean ± standard errors.

3.3.7 Statistical analysis

GraphPad Prism software (Version 7.0) was used to carry out all the data analyses. Statistical differences between groups were measured using Two-way analysis of variance (ANOVA) and Tukey's post-hoc procedure for simulated digestion while, for the immunomodulation assay One-way ANOVA was applied. T-test was applied to the adhesion assay results to determine statistical differences between *B. coagulans* and the *L. acidophilus* control and to the cytotoxicity assay results to confirm statistical differences between the control cell lines and *B. coagulans*. Data are expressed as mean ± standard errors (SEM) calculated over three independent experiments performed in triplicate. The differences between means were considered significant when p value < 0.05 (ns: non-significant).

3.4 Results

3.4.1 Survival of B. coagulans spores following in-vitro simulated digestion

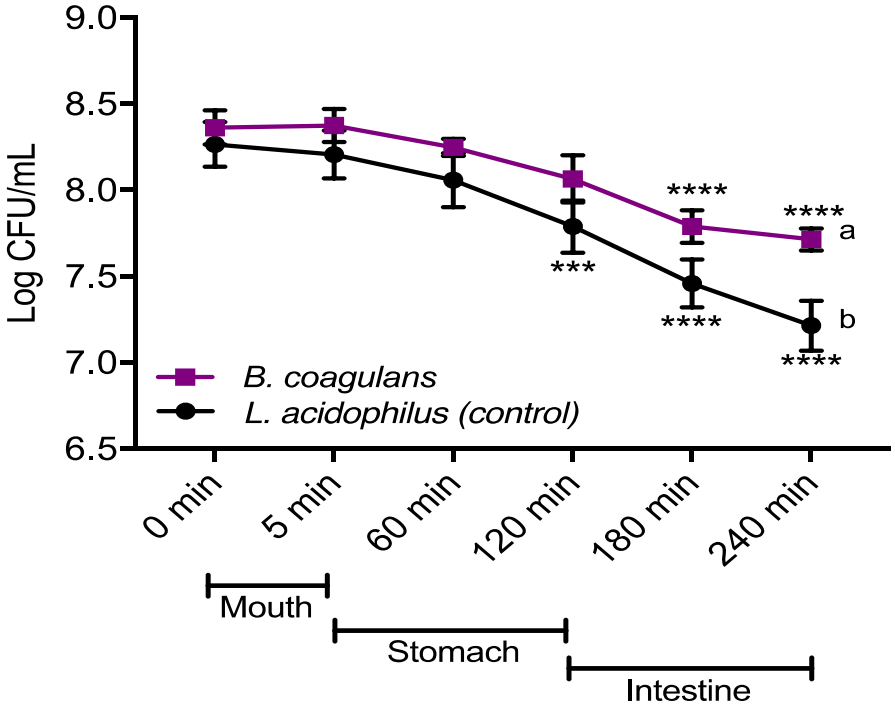


Figure 3.2. Survival of *B. coagulans* spores in the three compartments of digestion simulated *in-vitro*: mouth, stomach and intestine. *L. acidophilus* served as a control. The samples were taken at each step and the viable number of CFU/mL was evaluated on respective agar medium. Values are means ± SEM of three replicate experiments. The values of groups designated with different letters are significantly different at 240 min. For overall survival of individual bacteria * $P < 0.05$ compared to 0 min (***$P < 0.001$, ****$P < 0.0001$).

The survival curves of *B. coagulans* spores and of the *L. acidophilus* control during simulated digestion process are shown in Figure 3.2. *B. coagulans* MTCC 5856 spores used in this study showed very high resistance to the conditions encountered during the simulated digestion process which was approximately five times greater compared with that of the *L. acidophilus* control. No significant decrease in *B. coagulans* spore count was detected after exposure to simulated mouth (SSJ) ($P > 0.99$) and gastric (SGJ) ($P = 0.06$) conditions compared with that of the initial inoculum. There was a significant decrease in *L. acidophilus* count at the end of gastric phase ($P = 0.0003$), with further significant drop ($P < 0.0001$) in the cell count of 1.03 Log CFU/mL at the end of intestinal phase. For *B. coagulans* spores, a drop of only 0.64 Log in viable spore count ($P < 0.0001$) was recorded at end in the simulated intestinal phase. Hence, the significant ($P = 0.02$) survival rate of 92.4 % of the spores,

compared with 87.6 % survival for *L. acidophilus*, following 240 min of simulated digestion demonstrates its substantial resistance to digestion.

3.4.2 Adhesion capacity of B. coagulans spores

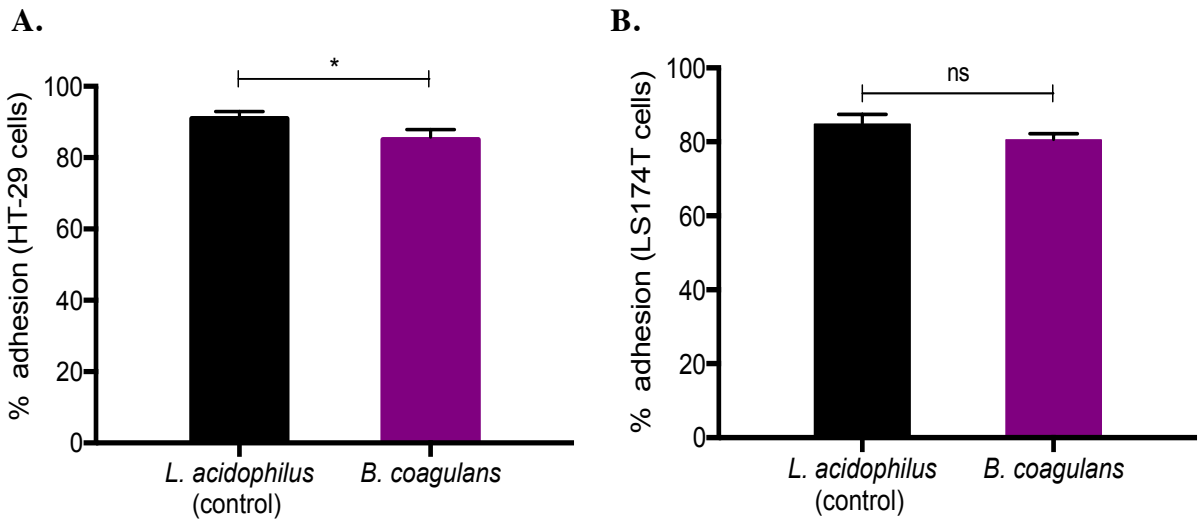


Figure 3.3 Adhesion of *B. coagulans* to (A) HT-29 and (B) LS174T cells after 4 h. Percentage of adherent *B. coagulans* spores and *L. acidophilus* (control) to HT-29 and LS174T cells after contact with bacterial suspensions for 4 h and washing of the cell monolayer. Values are means ± SEM of three replicate experiments.

The adhesion assay confirmed the ability of *B. coagulans* spores to adhere to both human colonic cells. Although, the *L. acidophilus* control showed significantly (P = 0.03) higher adhesion capacity to HT-29 cells (91.7 ±1.3%), *B. coagulans* spores also showed excellent adhesion at a rate of 85.8 ± 2.1 % (Figure 3.3A). In LS174T cells (Figure 3.3B) however, both *L. acidophilus* and *B. coagulans* showed statistically equal adhesion capacity (P = 0.14) with adhesion rates of 85.07 ± 2.4 % and 80.6 ± 1.45% respectively. No significant difference (P = 0.08) was observed between the cells lines for their capacity to support adhesion of *B. coagulans* spores.

3.4.3 Cytotoxicity analysis

The cytotoxic effect of spores on the HT-29 and LS174T cells was investigated after 8 h treatment with *B. coagulans* spores (Figure 3.4). The results showed that *B. coagulans* MTCC 5856 spores had no negative influence on viability of either of the colonic cell lines. An increase of extracellular LDH, which is indicative of cell membrane damage and cell death, was not observed for HT-29 (Figure 3.4A) or LS174T cells (Figure 3.4B) treated with spores

compared with their controls (p = 0.86, 0.67 respectively). Moreover, the treatment with spores was found not to result in any loss of viability in HT-29 cells (Figure 3.4C) or LS174T cells (Figure 3.4D) as confirmed by the Trypan blue exclusion test. These results show that *B. coagulans* MTCC 5856 spores do not exert cytotoxic effects on human colonic cell lines.

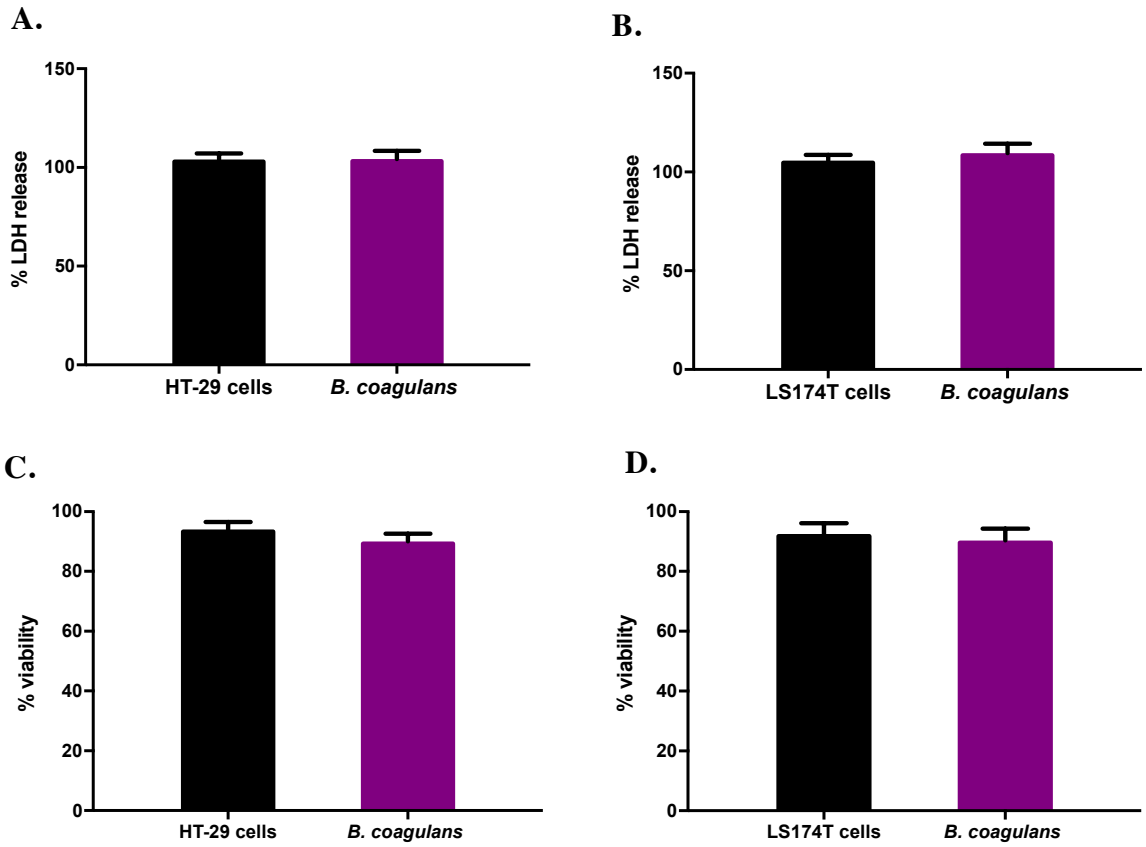


Figure 3.4. Effect of *B. coagulans* spores on cell viability. Percentage LDH release in **(A)** HT29 cells and **(B)** LS174T cells. Viability of cells represented as mean % LDH release after 8h treatment *with B. coagulans* spores versus untreated control. Viability of **(C)** HT-29 and **(D)** LS174T cells as determined by Trypan blue exclusion test and is represented as % viable cells remaining after 8 h treatment with *B. coagulans* spores versus untreated control. Values are means ± SEM of three replicate experiments.

3.4.4 Immunomodulatory effect of B. coagulans spores

The cytokine assay showed that *B. coagulans* MTCC 5856 spores led to significant reduction in the release of the pro-inflammatory cytokine IL-8 under normal and LPS-stimulated conditions (Figure 3.5A). HT-29 cells treated with *B. coagulans* MTCC 5856 markedly reduced ($P < 0.0001$) the IL-8 levels (114.8 pg/mL) compared with that secreted by HT-29 (negative control) cells (584.73 pg/ml). LPS stimulation of HT-29 cells induced secretion of high concentrations of pro-inflammatory IL-8 in HT-29 cells (1265 pg/mL). In

comparison with the positive control of LPS-stimulated cells, IL-8 secretion was substantially reduced by the co-treatment (69.24 pg/mL), and the post-treatment (641.1 pg/mL) of HT-29 cells with *B. coagulans* MTCC 5856 spores. A significant reduction (P= 0.0018) of IL-8 secretion was noted under the co-treatment condition relative to the post-treatment condition.

B. coagulans MTCC 5856 displayed significant ability to induce anti-inflammatory IL-10 secretion by HT-29 cells in both inflamed and non-inflamed conditions (Figure 3.5B). In comparison with the control HT-29 cells (3.11 pg/mL), the IL-10 secretion was markedly elevated ($P < 0.0001$) in non-inflamed cells treated with *B. coagulans* MTCC 5856 spores (19.88 pg/mL). Co-treatment of LPS-stimulated cells with *B. coagulans* MTCC 5856 (23.90 pg/mL) significantly ($P < 0.0001$) induced the secretion of higher concentrations of IL-10 compared to that secreted by non-treated LPS-inflamed cells (3.23 pg/mL). No IL-10 secretion was detected under the post-treatment condition ($P = 0.08$). Co-treatment with *B. coagulans* MTCC 5856 was noted to be more efficient compared with the post-treatment in modulating pro-inflammatory IL-8 and anti-inflammatory IL-10 secretions.

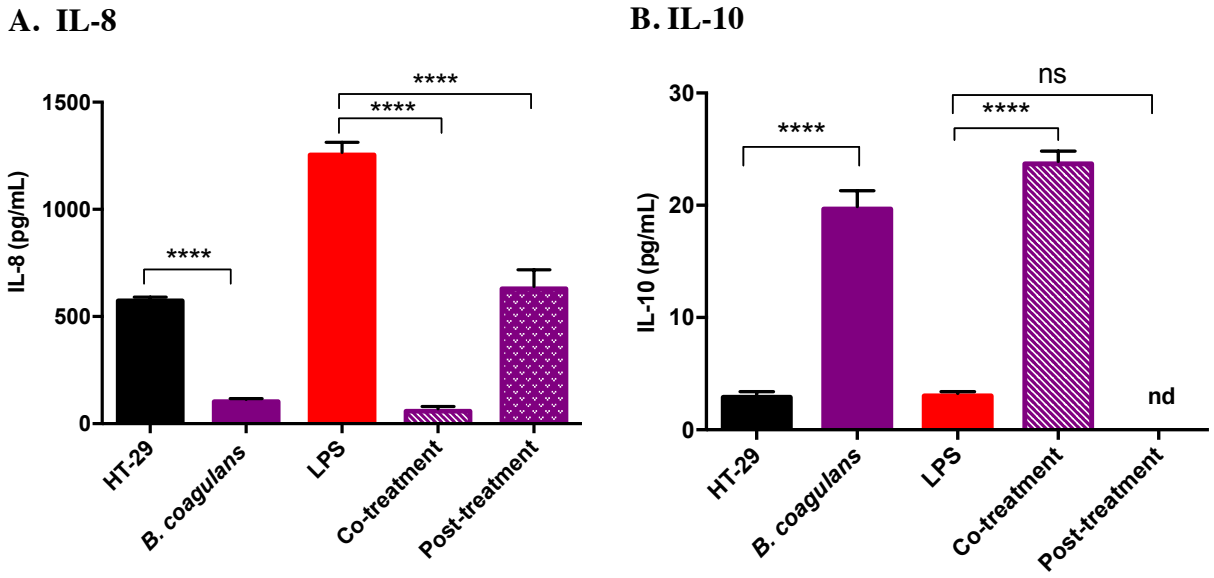


Figure 3.5. Quantification of cytokines secreted in the supernatant of HT-29 cells after treatment with *B. coagulans* spores by Bioplex assay. Cytokines **(A)** IL-8**, (B)** IL-10 released by HT-29 cells (negative control), *B. coagulans* treated HT-29 cells, LPS-stimulated HT-29 cells (positive control) and LPS-stimulated and treated with probiotic *B. coagulans* (co- and post-treatment). Data are represented as mean ± SEM of three repeated measurements. (nd= non-detected, ns= non-significant, **** $P < 0.0001$).

3.5 Discussion

This study has investigated four functional aspects of the probiotic potential of *B. coagulans* MTCC 5856 spores: 1) ability to survive during simulated digestion, 2) adhesion capacity, 3) safety and 4) immunomodulation activity (Figure 3.1). The *in-vitro* simulated digestion process was conducted to mimic the conditions of the human gastrointestinal tract after food ingestion by sequentially exposing *B. coagulans* spores and *L. acidophilus* (control) to acidic pH, bile salts and digestive enzymes encountered during gastric transit. *B. coagulans* spores showed excellent resistance to the simulated digestion process with a higher survival rate (~ 92.4%) at the end of 240 min digestion compared with the *L. acidophilus* control (~ 87.6%). The resistance to the digestion process is crucial since it ensures that the ingested dose actually reaches the gastrointestinal tract (GIT) where it essentially exerts its effects (458). The spores showed significant resistance to acidity (pH 3) and pepsin during their exposure in the gastric step of the *in-vitro* digestion. These results are in agreement with a recent study that reported good survival of *B. coagulans* MTCC 5856 in highly acidic pH (pH 1.5 and 3) conditions and growth on MRS agar containing bile salts (0.3% and 0.5% *w/v*) (44). In addition to the affect of acidity and bile, our study also demonstrated the excellent resistance of *B. coagulans* spores to digestive enzymes. The exposure to digestive enzymes (pepsin and pancreatin) did not affect the spores, which remained at relatively the same population at the end of the simulated digestion process. These results collectively indicate that a relatively large proportion of ingested *B. coagulans* MTCC 5856 spores can reach the colon unaffected by gastric acids, bile salts and enzymes.

A reduction in spore count of less than one logarithmic unit was observed in the intestinal phase of the simulation digestion (Figure 3.2). The possible reason for this small loss of spore count could be due to the acid activation of spore germination and subsequent killing of vegetative cells by simulated gastric and later by simulated intestinal fluids (459, 460). A hardened coating primarily consisting of integument proteins, is thought to protect the spores against gastric acid and bile salts (45). However, not all *Bacillus* spores are equally resistant to gastric transit (459). Hence, it is imperative to determine the survival of spores during digestion prior to their application as a probiotic. The greater than 90% survival of *B. coagulans* MTCC 5856 spores in our study emphasises the probiotic potential of this strain. Application of spores of *B. coagulans* MTCC 5856 for probiotic uses provides practical advantages as their incorporation does not require encapsulation or consideration of protection from food matrices since the spores are resistant to food processing temperatures, storage and

the hostile GIT environment. Thus, *B. coagulans* spores can serve in formulating shelf-stable foods as well as products that require higher temperature processing.

Adhesion to intestinal epithelium is a preferred attribute for a probiotic, as it will ensure, at least transiently, the colonisation of mucosal surfaces, thus facilitating interference with pathogen binding and bacterial interaction with immune cells (461). Similar to *L. acidophilus* control, *B. coagulans* spores used in our study displayed remarkable adhesiveness towards both human mucus-secreting colonic cells HT-29 (82.92%) and LS174T (79.34%). These results correlate with that of Ripamonti et al. (462) who reported adhesion of *B. coagulans* (isolated from calf faeces) to INT407 cell monolayers via microscopy following Giemsa staining. The substantial adhesion capacity of *B. coagulans* MTCC 5856 spores to intestinal cells therefore demonstrates that *B. coagulans* MTCC 5856 has an attribute that can enhance probiotic efficacy.

Different studies have highlighted the suitability of human intestinal cell-lines including HT-29, LS174T and Caco-2 cells, as *in-vitro* model systems for examining the colonisation capacity of bacterial strains to colonic cells (452, 463-465). HT-29 and LS174T cells have been reported to possess substantial mucin secretion ability with significant Muc2 mRNA expression compared with Caco-2 cells (466). Previous studies showed preferred bacterial adhesion to Muc2 expressing cells suggesting a vital role of mucin in bacterial adhesion (467, 468). The significant adhesion of *B. coagulans* spores to colonic HT-29 and LS174T cells in our study could be attributed to high adhesiveness to mucins present in the native human mucus layer covering the whole cell surface.

It is known that both the spore coat and the exosporium main protein component of the spore have prominent roles in spore adhesion (469, 470). Probiotic spores of *B. cereus* were found to be more adhesive to Caco-2 cells and mucin than the vegetative cells by Sánchez et al. (471) suggesting the role of spore-coat–associated proteins in the interaction with intestinal epithelial cells within the gastrointestinal tract. Hydrophobicity of spores, as well as spore surface appendages, may also play an important role in their adhesive capacity to the hydrophilic intestinal mucus layer (472, 473). *B. coagulans* spores have been reported to exhibit 54% of hydrophobicity by Mohkam et al. (474). Therefore, high adhesion rates observed in our study for *B. coagulans* MTCC5856 spores could involve the role of several mechanistic factors and needs further investigation.

Bacterial adhesion reflects the potential colonisation by the probiotic in the GIT that may prevent pathogens from attaching via specific obstruction on cell receptors or steric interactions (475). *B. coagulans* spores have been proven effective in reducing colonisation of vancomycin-resistant *Enterococcus* in mice (476). Recently, *B. coagulans* MTCC 5856 have been shown to exhibit inhibitory potential against *E. coli* (477, 478). Thus, the adhesion of *B. coagulans* to intestinal cells could be an important contribution in mediating competitive pathogen exclusion and communication with the immune system. In this study, the remarkable ability of *B. coagulans* MTCC 5856 to adhere to human colonic cells further corroborates their probiotic potential.

The safety of probiotic strains is given prime importance in the selection process of probiotics (479). In terms of safety of *Bacillus* species, with the exception of *Bacillus anthracis* and *Bacillus cereus*, *Bacillus* species are not generally considered pathogenic (480). However, if the spores are to be consumed in large quantities on a regular basis as probiotics, a safety evaluation for each strain is vital. In our study, *B. coagulans* MTCC 5856 spores at a dose of 2×10^9 CFU/ mL, did not exhibit cytotoxic effects on HT-29 or LS174T cells as confirmed by LDH assay and Trypan blue exclusion test. This strengthens the probiotic grading of *B. coagulans* MTCC 5856 for food applications. The FDA (2015) confirmed a "generally regarded as safe" (GRAS) status to *B. coagulans* MTCC 5856 (LactoSpore®) spore preparation, further supporting its application in a variety of foods. Moreover, double-blind, placebo-controlled studies verified that 30-day and 90-day supplementation of *B. coagulans* MTCC 5856 (at a dose of 2×10^9 CFU spores/day) was evaluated as safe and tolerable in healthy human participants with supplementation (69) and improved quality of life and decreased irritable bowel syndrome symptoms (68).

Immunomodulatory capacity of probiotic bacteria is also one of the vital criteria for the assessment of a probiotic strain (481) and for grading its probiotic potential. Even though the metabolically inactive nature of spores raises questions on their immunomodulatory capacity, a number of studies have demonstrated the ability of *Bacillus* spores to interact with immune system (381, 459, 482). Previous reports have also shown that the spore itself is immunomodulatory and can trigger a number of cellular immune responses in the GIT. This is consistent with the observations of our study (409, 482-484). *B. coagulans* MTCC 5856, which was in their spore form in our study, exhibited substantial immunomodulatory efficacy suggesting potent immunogenic capability of the spore form.

Low-grade inflammation, loss of microbial diversity and outgrowth of Gram-negative bacteria are the hallmarks of inflammatory gastro-intestinal disorders including IBD. Therefore, the efficacy of *B. coagulans* MTCC 5856 spores to moderate the inflammatory reaction of the LPS–stimulated HT-29 cells was investigated in this study by evaluating the secretion of key anti-inflammatory and pro-inflammatory cytokines. Binding of LPS to Toll-like receptors (TLRs), induces intestinal inflammation via generation and overproduction of pro-inflammatory cytokines including IL-8 (485, 486). In our study, under the influence of LPS, pro-inflammatory cytokine IL-8 was found to be secreted at comparatively higher levels but markedly reduced by co- and post-treatment with *B. coagulans* spores. The increase in pro-inflammatory cytokine secretion induced by LPS could be attributed to T cell proliferation and activation triggered by LPS-TLR interaction (487). The reduction in the pro-inflammatory cytokine, IL-8 after treatment with *B. coagulans* MTCC 5856, could potentially be elicited by their ability to interfere with IL-8/LPS signalling and subsequently hindering the molecular events leading to T cell activation.

Interestingly, *B. coagulans* MTCC 5856 spores in our study, showed a significant reduction in IL-8 secretion under both co- and post-treatment conditions (Figure 3.5A), with more effective reduction under co-treatment of LPS-stimulated HT-29 cells. This differential effect could possibly be attributed to different signalling pathways induced by probiotic spores under different inflammatory conditions. Moreover, this differential immunomodulatory effect highlights the prudence of time of application of probiotic spores to contain the colonic inflammation. This is in agreement with Azimirad et al. (394), who observed a similar differential immunomodulatory effect in a different strain of *B. coagulans* with substantial reduction in secretion and mRNA expression of IL-8 under pre-treatment, but no effect was observed under post-treatment in LPS-induced HT-29 cells. The inhibition of pro-inflammatory response by probiotic *Bacillus* spores could be associated with their capacity to interfere with IL-8/LPS signalling. Interaction of some spore-coat ligands of *Bacillus* probiotics with immune receptors (e.g. TLRs) on the intestinal epithelial cells could promote an immunomodulatory effect on the inflamed GIT in response to enteric pathogens or their antigens. De Souza et al. (488) demonstrated the interaction of *B. subtilis* spores with TLRs. Interaction of *Bacillus* spore-specific ligands with TLRs during co-treatment condition may hinder the LPS-TLRs interaction, that in turn modulate the induction of IL-8 in intestinal cells. Such an antagonistic response, that benefits by inhibiting the bacterial-induced inflammation in the intestinal cells, was established by a probiotic *Lactobacillus* strain (489).

The remarkable immunomodulatory capacity of *B. coagulans* MTCC 5856 spores is indicated by its ability to induce increased secretion of anti-inflammatory cytokine IL-10 while concurrently reducing the secretion of pro-inflammatory cytokine IL-8. Our results appear to be consistent with findings of several other investigators who also reported considerable reduction in IL-8 (452, 455, 490-492) and increase in IL-10 (452, 455) using probiotic treatment under *in-vitro* conditions with different strains of lactic acid bacteria (LAB) and different inflammatory agents. Previously, LPS-stimulated peripheral blood mononuclear cells PBMCs from healthy elderly patients showed a 0.2 ng/mL increase in IL-10 28 days after consumption of *B. coagulans* (409). In another study, cell wall and metabolite fractions of *B. coagulans* spores inhibited the polymorphonuclear leukocyte migration towards IL-8 as well as enhanced mitogen-induced expression of IL-10 *in-vitro*. Both components of *B. coagulans* modulated the production of cytokines in that they inhibited IL-2 production, improved production of IL-6, IL-4 and IL-10 (429).

Under the non-LPS condition, that represented a normal physiological condition, *B. coagulans* spores showed remarkable efficacy to increase IL-10 secretion compared to the basal level of control HT-29 cells. In addition, contact with *B. coagulans* spores reduced the secretion of IL-8 relative to that of control HT-29 cells, making them excellent probiotic candidates for application in gut immune homeostasis. *B. coagulans* spores were found to be effective in elevating the secretion of anti-inflammatory IL-10 in colonocytes under non-inflamed and LPS-stimulated-co-treated conditions in this study. However, under post-treatment conditions, where LPS previously inflamed HT-29 cells, IL-10 levels could not be detected. The inability of *B. coagulans* spores to induce IL-10 secretion in already inflamed cells (post-treatment) strongly suggests that the time of probiotic treatment could influence the cytokine regulation in HT-29 cells. This is in agreement with Duary et al. (455) who also observed inability of *Lactobacillus* strains to increase the IL-10 gene expression levels during post-treatment of previously LPS-stimulated HT-29 cells relative to pre- and co-treatments. This effect was arbitrarily attributed to the involvement in different signal pathways triggered by the same probiotic strain under different conditions (455). Based on the analysis of IL-8 and IL-10 secretions on the differently exerted immunomodulatory benefits under different (inflamed and non-inflamed) conditions in our study, the application of *B. coagulans* spores before (as prophylactic agent) or during (therapeutic) the onset of inflammation is critical in order to acquire optimum benefits from *B. coagulans* spores.

To gain clear understanding of different immune signalling pathways utilised by probiotic spores under different conditions (co-, post-inflamed and non-inflamed) more *in-vitro* and *in-vivo* studies focussing on these aspects are required. Pronounced ability of *B. coagulans* MTCC 5856 spores to induce the immunomodulatory effects showcased in our study strengthens the assessment of its probiotic effectiveness.

3.6 Conclusions

It can be concluded from this study, that *Bacillus coagulans* MTCC 5856 spores demonstrated considerable probiotic potential. These spores showed substantial ability to survive the gastric and intestinal conditions and then to colonize the intestine, at least temporarily, by effectively adhering to the colonic epithelium in equal comparison with a probiotic *L. acidophilus* control. Moreover, the spores did not show any cytotoxic effects towards HT-29 or LS174T cells but, did exhibit excellent immunomodulatory efficacy by downregulating the secretion of the key pro-inflammatory cytokine IL-8 while concomitantly promoting increased secretion of anti-inflammatory cytokine IL-10. More importantly, these spores also exhibited remarkable immunomodulatory and anti-inflammatory potential in HT-29 cells under co-treatment relative to post-treatment condition highlighting the value of early application of these probiotic spores in order to suppress inflammation. By virtue of possessing these prophylactic and therapeutic attributes, applications of these probiotic spores can be explored as potential therapeutic agents in the management of intestinal inflammatory disorders including IBD and as adjunct therapeutics through their immune-stimulatory action. The excellent survival during digestion, adhesion capacity and marked immunomodulatory efficacy of *B. coagulans* MTCC 5856 spores coupled with their known ability to survive the food processing techniques and storage supports their incorporation into novel shelf stable food products targeted at improving and/or treating gut health.

Chapter 4

Synbiotic supplementation containing whole plant sugar cane fibre and probiotic spores potentiates protective synergistic effects in mouse model of IBD

4.1 Abstract

IBD are chronic inflammatory disorders with increasing global incidence. Synbiotic supplementation, which is a two-point remediation approach carrying probiotic and prebiotic components for mitigating inflammation in IBD, is thought to be a pragmatic tactic owing to possible synergistic outcomes. In this study, the impacts of dietary supplementation with probiotic *Bacillus coagulans* MTCC5856 spores (*B. coagulans*) and prebiotic whole plant sugar cane fibre (PSCF) was assessed using a murine model of IBD. Eight-week-old C57BL/6 mice were fed a normal chow diet supplemented with either *B. coagulans*, PSCF or its synbiotic combination. After seven days of supplementation, colitis was induced with dextran sulfate sodium (DSS) in drinking water for seven days during the continuation of the supplemented diets. Synbiotic supplementation ameliorated disease activity index and histological score (−72%, 7.38, respectively), more effectively than either *B. coagulans* (−47%, 10.1) and PSCF (−53%, 13.0) alone. Synbiotic supplementation also significantly ($p < 0.0001$) prevented the expression of tight junction proteins and modulated the altered serum IL-1β (−40%), IL-10 (+26%), and C-reactive protein (CRP) (−39%) levels. Synbiotic supplementation also raised the short-chain fatty acids (SCFAs) profile more extensively compared to the unsupplemented DSS-control. The synbiotic health outcome effect of the probiotic and prebiotic combinations may be associated with a synergistic interaction of the direct immune-regulating efficacy of the components, their ability to protect epithelial integrity, stimulation of probiotic spores by the prebiotic fibre, and/or with stimulation of greater levels of fermentation of fibres releasing SCFAs that mediate the reduction in colonic inflammation. The model findings suggest synbiotic supplementation should be tested in clinical trials and supports the justification for their incorporation into functional foods for improved gut health.

4.2 Introduction

IBD are chronic relapsing inflammatory conditions of the gastrointestinal tract that comprises two partially overlapping but distinct clinical entities: Crohn's disease (CD) that involves the entire gastrointestinal tract and ulcerative colitis (UC) that is limited to colon and rectum (19). The incidence of CD and UC has become of global significance with accelerating occurrence in countries adopting a Westernised diet, highlighting the urgent need for research into prevention and management of this complex and costly pathology (493). Although the aetiology and pathogenesis of IBD still remains unclear, emerging evidence supports the involvement of a recurrent tripartite pathophysiological circuit encompassing gut dysbiosis, altered epithelial integrity and defective immune responses (19). Therefore, preventive and therapeutic approaches that impede or break this inflammatory circuit by resolving one or more of the pathophysiological circuit components are highly sought.

Dietary interventions are increasingly perceived as both preventive and corrective strategies to normalise the dysfunctional microbiome and altered immune and barrier integrity functions in IBD (27, 60, 494). In this regard, probiotic and prebiotic DFs are thought to be useful in mitigating the inflammatory circuit thereby resolving or preventing the severity of IBD. Both bioactive ingredients can improve inflammatory parameters in the gut by modifying microbiota composition and metabolites, regulating secretion of immunomodulatory molecules and protecting the colonic epithelial barrier (19, 26, 28, 495). Synbiotics, being a combination of probiotic and prebiotic ingredients that positively interact, potentially offer prophylactic and therapeutic effects that could function synergistically to confer health benefits to the host.

DFs have shown particular promise in attenuating colonic inflammation in humans (11). The underlying mechanisms of effectiveness are likely to be multifactorial including dilution of toxins via stool bulking and the production of metabolites, particularly SCFAs, as a result of microbial fermentation. This later mechanism is frequently cited as a major potential contributor to the protective effect (23). DFs consist of edible plant parts that resist digestion and absorption in the small intestine and undergo complete or partial fermentation in the colon. It is an extremely complex group of substances, including non-starch polysaccharides, resistant starch, cellulose and hemicellulose, oligosaccharides, pectins, gums, lignin, and waxes (496). Much work on DFs, however, has examined various purified ingredients that represent limited chemical complexity, contrasting to those that naturally occur in fruits and

vegetables (52). Nevertheless, the biochemical complexity of DFs is recently being more appreciated to be a vital factor influencing the microbial complexity of the gut (52-54). This highlights the prudence of applications using prebiotic fibres that are representative of whole plant vegetables and fruits which retain fibre biochemical complexity compared to plant fibres that are extracted and potentially purified fractions. In this context, a process to produce sugar cane fibre by wet diffusion to remove most of the sucrose from cut cane, which is then dried and ground into a flour, has been reported to preserve the cell wall components (73, 74). Such fibre, in addition to retaining other intrinsic nutritional biologically active components, such as micronutrients and polyphenols, also contains both soluble and insoluble fibre benefits. These materials are comprised mainly of cell wall components and have a mix of rapid- and poor-fermentable fibres and at ratios that more accurately represent natural whole plant foods. In a recent study, such sugar cane fibre has been shown to impart positive effects on human gut microbiota *in-vitro* (75). The high content of total dietary fibre (87%) was accounted for with respect to its positive effect in this study. The relative similarity of the fibre components of this whole plant sugar cane fibre product to that in other whole plant foods, and the lack of any significant sugar or starch content, indicates its potential as a convenient supplementary dietary source of fibre. The availability of the cell wall components for fermentation in the lower bowel could alter microbial ecology and have a positive influence for IBD attenuation.

Bacillus coagulans is a GRAS (generally recognised as safe) affirmed probiotic that can ferment a variety of plant substrates rich in insoluble cell wall components (64, 71) more efficiently than most members of gut microbiota (497, 498). It is also known to be capable of modulating the innate immune system by binding and interacting with the gastrointestinal tract epithelium (42, 432). This makes it a suitable probiotic for its synbiotic combination with prebiotic PSCF rich in insoluble cell wall fractions. Probiotic *Bacillus coagulans* MTCC (Microbial Type Culture Collection) 5856 spores, in addition to exhibiting excellent immunomodulatory effects *in-vitro*, have shown significant survival during simulated gastric transit with substantial adhesion capacity to human colonic epithelial cells (42). Based on the aforementioned findings, we hypothesised that preconditioning with probiotic *B. coagulans* MTCC 5856 spores, prebiotic PSCF or their synbiotic combination might repress the onset and/or severity of DSS-induced colitis in mice. This study therefore aimed to evaluate the efficacy of probiotic *B. coagulans* spores and PSCF, both alone and in combination, as a synbiotic dietary supplement to ameliorate the onset of experimental colitis in mice and further examine its underlying mechanisms of efficacy.

4.3 Materials and methods

4.3.1 Probiotic Bacteria and Prebiotic Dietary Fibre

LactoSpore® (Sabinsa Corporation, East Windsor, NJ, USA) containing the probiotic strain *Bacillus coagulans* MTCC 5856 (6×10^9 spores/gm) was produced by Sami Labs Limited (Bangalore, India) and supplied by Sabinsa Corporation (Australia). Kfibre™, PSCF was supplied by KFSU Pty Ltd., Queensland, Australia (Appendix I).

4.3.2 Animals

Fifty C57BL/6J (seven week old) mice of both sexes of average weight 19g were obtained from the University of Tasmania animal breeding facility and housed in a temperature-controlled environment with a 12-h day/night light cycle. Individual body weights were assessed daily including over an initial acclimation period of seven days. All mice had *ad libitum* access to radiation-sterilised rodent feed pellets (Barastoc Rat and Mouse, Ridley AgProducts, Australia, Appendix III) and autoclaved tap water for drinking during experiments. All animal experiments were approved by the Animal Ethics Committee of the University of Tasmania [ethics approval number: A0015840 (Appendix IV)] and conducted in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (8th Edition, 2013). All efforts were made to minimize animal suffering and to reduce the number of animals used.

4.3.3 Study Design and Treatments

Following one week of acclimation, mice at eight weeks of age were randomly allocated into the following five groups (n = 10 per group): (1) Healthy control (HC), (2) DSS-control, (3) probiotic *B. coagulans* MTCC 5856 spores (*B. coagulans*), (4) whole plant prebiotic sugar cane fibre (PSCF) supplement, and (5) synbiotic combined supplement (Synbiotic). Figure 4.1 illustrates the experimental design of the mice feeding trial. Mice in HC and DSS-control groups received 4 g chow mash (standard chow pellet blended with water). The *B. coagulans* group received 4 g chow mash supplemented with probiotic *B. coagulans* MTCC 5856 spores (2×10^9 CFU/day/mouse). The PSCF group received 4g chow mash supplemented with Kfibre™ (200 mg/day/mouse). The Synbiotic treatment group mice received 4 g chow, each supplemented with *B. coagulans* MTCC 5856 spores (2×10^9 CFU/ day/mouse) and Kfibre (200 mg/day/mouse). The chow mash was prepared fresh each day. The mice were single-caged throughout the experiment to ensure the defined daily intake of respective treatments

from prepared chow mash. The mice were fed these treatments for 14 days. Colitis was induced during the last seven days of the experimental period as previously described (499), by administering 2% dextran sulfate sodium (DSS; MP Biomedicals, colitis grade average molecular weight: 36,000–50,000) in the drinking water of all groups except for the non-colitic DSS-control mice which received normal drinking water. Mice were sacrificed on day 15 by CO_2 asphyxiation.

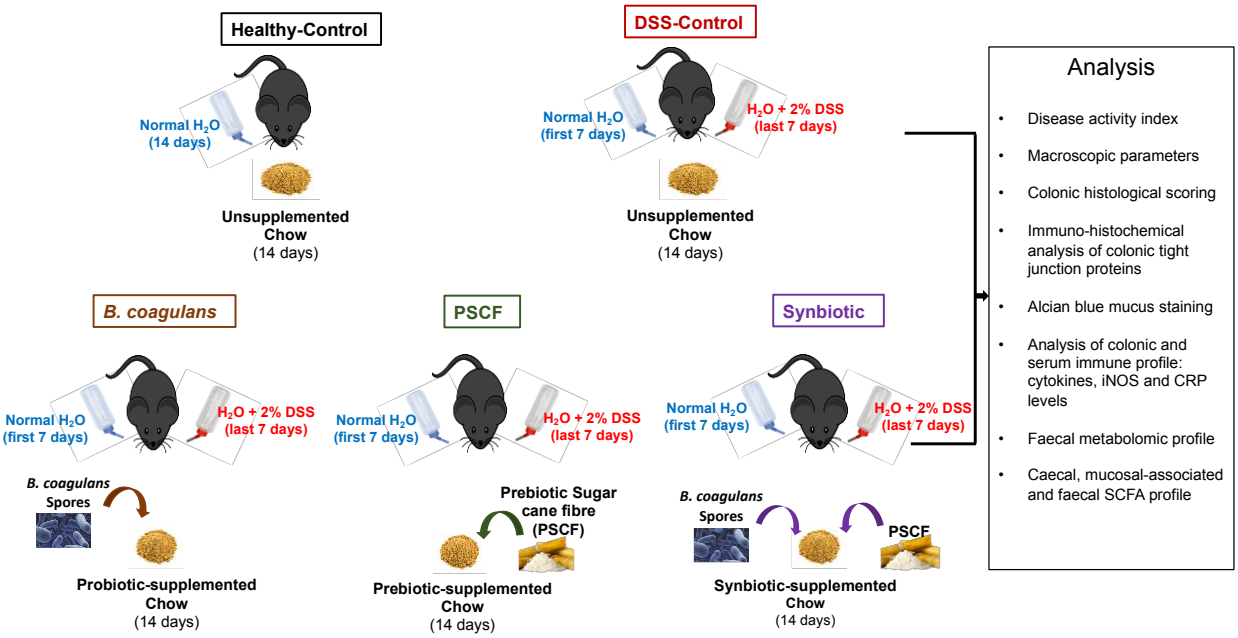


Figure 4.1. Experimental design of *in-vivo* feeding trial to analyse prophylactic efficacy of *B. coagulans* spores, PSCF and Synbiotic in DSS-induced acute colitis mice model. C57BL/6J mice (n = 10 per group) were fed chow supplemented with either *B. coagulans* spores, PSCF or their Synbiotic combination for 14 days. Colitis was induced by administration of 2% DSS in drinking water for last seven days.

4.3.4 Clinical Scoring and Histological Analysis

A Disease Activity Index (DAI) was determined daily in all mice by scoring for body weight, hemocult reactivity or presence of gross blood and stool consistency during the week of DSS induction, as detailed in (500). Stool was collected from individual mice and tested for the presence of blood using Hemoccult II slides (Beckman Coulter Inc., Brea, CA, USA). Briefly, the following parameters were used for calculation: (a) body weight loss (score 0 = 0%, score 1 = 1–5%, score 2 = 6–10%, score 3 = 11–15%); (b) stool consistency (score 0 = normal, score 1 = soft but still formed, score 2 = very soft/loose stool, score 3 = diarrhoea/watery stool); and (c) blood in stool (score 0 = negative hemoccult, score 1 = positive hemoccult, score 2 = blood traces in stool visible, score 3 = rectal bleeding). DAI was

determined by combining the scores from these three categories. Body weights were measured for each animal throughout the experiments and expressed as percent weight loss to the weight immediately before DSS treatment. Faecal samples were collected and stored at −80 °C on day 14 for metabolite analysis.

After sacrificing the mice, the colons were removed from the caecum to the anus following the method of Perera et al. (501). The length of the colons from the ileocaecal junction to the rectum were recorded. The colon was subsequently opened along its longitudinal axis and the luminal (mucosal) contents were removed using sterilised 200 μL pipette tips prior to weighing the organ. The length and weight of colon and spleen were documented. Spleen weight, colon length, and colon weight/body weight ratio were calculated as macroscopic markers of inflammation. The contents of colon (mucosal-associated) and caecum were collected by scraping with sterile pipette tips for metabolite profiling and stored at −80 °C. The colon was bisected longitudinally, and one half was prepared using the Swiss roll technique (502) whereas the remaining colonic tissue was dissected out, segregated into proximal colon (PC) and distal colon (DC) and snap-frozen for molecular analyses. Swiss rolls underwent 24 h fixation in 10% (v/v) neutral-buffered formalin. Swiss rolls were subsequently transferred to 70% ethanol prior to progressive dehydration, clearing and infiltration with HistoPrep paraffin wax (Fisher Scientific, Philadelphia, PA, USA). Swiss rolls were then embedded in wax and 5 μm sections were cut using a rotary microtome. Sections were stained with haematoxylin and eosin (H and E; HD Scientific, Sydney, Australia). Slides stained with H&E (n = 8 per group) graded blindly for the severity of tissue damage at distal and proximal regions as described previously (503, 504). Briefly, frequency of distribution of inflammation graded 0-3, crypt architectural distortion and ulceration graded 0–5, tissue damage graded 0-3, inflammatory infiltrate graded 0–3, goblet cell loss graded 0–3, mucosal thickening (oedema) were graded 0–3. All images were captured on a Leica DM500 microscope using a Leica ICC50 W camera (Leica Microsystems, Wetzlar, Germany).

4.3.5 Alcian Blue Staining

DSS-induced alterations in goblet cells, and subsequent depletion in synthesis and secretion of mucin glycoprotein (MUC2) was analysed by Alcian blue staining (ab150662 Alcian Blue, pH 2.5 (Mucin Stain), Abcam, Australia) following the manufacturer's instructions. Briefly, paraffin-embedded colon sections (n = 4/group) were stained with

Alcian blue, resulting in staining of the acidic sulphated mucin blue and the counterstained with Safranin O, staining the nuclei red following the method previously described (505). Computer-assisted image analysis was performed with a Leica DM500 microscope (Leica Microsystems, Wetzlar, Germany) and Leica ICC50 W camera (Leica Microsystems, Wetzlar, Germany). The staining intensity (IOD) was assessed using Image Pro Plus 7.0 (Media Cybernetics, Inc., Rockville, MD, USA) and used for comparison among groups (506).

4.3.6 Immunohistochemical Detection of Tight Junction Proteins

Immunohistochemical detection of epithelial tight junction (TJ) proteins was performed using a Rabbit specific HRP/DAB (ABC) Detection IHC kit (ab64261, Abcam, Australia) following the manufacturer's instruction, and as previously described (507). Following removal of paraffin and rehydration, the tissue sections were exposed to heat-induced epitope retrieval (4 min at 121 °C) in a sodium citrate buffer, pH 6 in a Decloaking chamber (Biocare Medical, Pachico, CA, USA). After washing the slides in 1× phosphate buffered saline (PBS) 2 mins/wash, endogenous peroxidase activity was blocked by incubating the slides with hydrogen peroxide block for 10 min. Next, the slides were washed with PBS (2 × 2 min) washes and protein block was then applied for 30 min at room temperature to block non-specific background staining. Following PBS (1 × 2 min) wash, colon sections were then incubated with primary antibodies: anti-ZO-1 (NBP1-85046, Novus Biologicals, Australia, 1:400); anti-occludin (NBP1-87402, Novus, 1:600); anti-claudin-1 (NBP1-77036, Novus, 1 μg/mL) overnight at 4 °C. Sections were then washed with PBS (4 × 2 min) and biotinylated goat anti-rabbit IgG was applied and incubated for 10 min at room temperature. At the end of incubation, the slides were washed in PBS (4 × 2 min) and streptavidin peroxidase was applied to the sections which were further incubated for 10 min at room temperature. The slides were then thoroughly rinsed with PBS (4 × 2 min) before sections were covered with 3,3'-diaminobenzidine (DAB) chromogen and substrate solution for 10 min. Tissue sections were subsequently counterstained with hematoxylin, dehydrated, and mounted with DPX media (Sigma-Aldrich, Sydney, Australia).

Computer-assisted image analysis was performed with a Leica DM500 microscope (Leica Microsystems, Wetzlar, Germany), Leica ICC50 W camera (Leica Microsystems, Wetzlar, Germany), and Image Pro Plus 7.0 (Media Cybernetics, Inc., Rockville, MD, USA) software. The expression of tight junction (TJ) proteins: ZO-1, occludin and claudin-1 was

blindly assessed by choosing random five fields on each slide (n = 4/group). Barrier TJ protein expressions and staining intensity in colonic epithelium was expressed as the percentage expression of a respective TJ protein.

4.3.7 Myeloperoxidase Activity

The extent of the inflammatory cell invasion in the colon was examined by the assessment of myeloperoxidase (MPO) activity (499). Weighed and snap frozen PC and DC specimens (n = 3) were analysed for MPO activity using a Myeloperoxidase Activity Assay kit (ab105136, colorimetric, Abcam®, Cambridge, UK). Briefly, frozen tissue after washing in cold PBS, was resuspended in MPO assay buffer, before homogenisation with 10–15 passes using an Omni TH tissue homogeniser (Omni International, US) with 10–15 passes. The homogenate was then centrifuged at 13,000× g (10 min) and the supernatant assayed for MPO activity as per the manufacturer's instructions. The values are expressed as MPO activity units/g tissue.

4.3.8 Tissue Explant Culture and Cytokine Measurements

PC and DC colon tissues of mice from each group were cut, weighed and washed with cold PBS before transferring to a 12-well plate containing 0.5 mL/well of RPMI1640 culture medium (In Vitro Technologies Pty Ltd, Melbourne, Australia) supplemented with 10% v/v foetal calf serum (Gibco, Life Technologies Pty Ltd, Melbourne, Australia), penicillin (100 mU/L), and streptomycin (100 mg/L) (Sigma-Aldrich Pty Ltd, Sydney, Australia) as described previously (501). After 24 h of incubation, supernatant was collected from each well, centrifuged and stored at −80 °C until further analysis. Serum was collected from blood drawn by cardiac puncture at the end of the study for cytokine analysis.

The cytokine levels in colon tissue (n = 3) and serum (n = 3) were determined by immunoassay using a Bio-Plex Pro Mouse Cytokine 23-plex kit (Bio-Rad #M60009RDPD, Bio-Rad Laboratories, Gladesville, NSW, Australia) following the manufacturer's instructions and concentrations analysed using a Bio-Plex 200 instrument (Bio-Rad) and Bioplex Manager software, version 6 (Bio-Rad Laboratories) respectively. For tissues, the cytokine levels were normalized by dividing the cytokine results (pg/mL) by the measured biopsy weight (g). The most significantly altered cytokines are presented as pg/g of tissue.

4.3.9 iNOS Activity

The expression of inducible isoforms of nitric oxide synthase (iNOS) in colonic epithelial cells, in response to pro-inflammatory stimuli (508), was determined in PC and DC specimens using a Nitric Oxide Synthase Activity Assay kit (ab211084, Fluorometric, Abcam®, Cambridge, UK). Snap frozen proximal and distal colonic tissues (n = 3) were washed in in cold PBS, resuspended in 200 μL NOS assay buffer, then homogenised by 10–15 passes of an Omni TH tissue homogeniser (Omni International, Tulsa, OK, USA). The homogenate was then centrifuged at 10,000× g (10 min, 4 °C) and the supernatant then underwent iNOS activity assay activity assay as per the manufacturer's instructions. The amount of protein in the lysate was determined using DC™ Protein Assay (Bio-Rad Laboratories, Australia). The results are expressed as iNOS activity mU/mg.

4.3.10 Serum C-Reactive Protein Analysis

The levels of C-reactive protein (CRP) in serum from respective groups (n = 3 samples/group) were analysed using Mouse C-Reactive Protein/CRP Quantikine Elisa kit (MCRP00, R and D Systems, Australia) following the manufacturer's instructions. The results are expressed as μg/mL.

4.3.11 Volatile SCFA Analysis

Volatile SCFA profiling of caecal, mucosal-associated and faecal samples were performed using GC-MS analysis by Dr. David J. Beale (CSIRO), Dr. Avinash V. Karpe (CSIRO) and Dr. Shakuntala V. Gondalia (Swinburne university of Technology). Data analysis and interpretation was performed by the PhD candidate. For the GC-MS analysis, caecal, mucosal-associated and faecal samples (n = 5 per group) were prepared and derivatized following the protocol developed by Furuhashi et al. (509) with some modifications. Briefly, caecal, mucosal-associated and faecal samples of 100–150 mg fresh weight (stored at −80 °C) were weighed to ± 0.1 mg accuracy. These samples were added to a sterile 1.5 mL bead-beating tube (NAVY Rino Lysis tubes, Next Advance, Troy, NY, USA). A 1.0 mL aliquot isobutanol (10% MilliQ water), (LC-MS grade, Merck, Castle Hill, NSW, Australia) was added to each sample, followed by two 30 s, 4000 rpm homogenization pulses sandwiched between a 20-s pause interval (Precellys Evolution Homogenizer, Bertin Instruments, Montigny-le-Bretonneux, France). The samples were subsequently centrifuged at 16,000× g for 6 min.

The supernatant (675 µL) was transferred to a clean round bottomed 2 mL centrifuge tube (Eppendorf South Pacific Pty. Ltd., Macquarie Park, NSW, Australia) and NaOH (20 mM, 125 µL, Merck Pty Ltd., Castle Hill, NSW, Australia) and chloroform (400 µL, LC-MS grade, Merck Pty Ltd.,) were added. The samples were briefly vortexed and centrifuged at 16,000·*g* for 3 min. The aqueous phase (upper layer, 400 µL) was transferred to a new clean round bottomed 2 mL centrifuge tube (Eppendorf South Pacific Pty. Ltd., Macquarie Park, NSW, Australia) containing a boiling chip (Sigma Aldrich, Castle Hill, NSW, Australia). Pyridine (100µL), isobutanol (80 µL) (both LC-MS grade, Sigma Aldrich, Castle Hill, NSW, Australia), and MilliQ (Millipore Corporation) water (70 µL) were added and the samples were subjected to gentle hand vortexing (swirling action) followed by the addition of 50 µL isobutyl chloroformate (98% purity, Sigma Aldrich, Castle Hill, NSW, Australia). The tube was kept opened to release any generated gases and was allowed to stand for about one minute. Hexane (150 µL, LC-MS grade, Sigma Aldrich, Castle Hill, NSW, Australia) was then added to each tube, which was then capped and vortexed prior to centrifugation at 15,700× *g* for 4 min. The upper phase (100 µL) was subsequently transferred to clean gas chromatography (GC) autosampler vial fitted with silanized low volume glass inserts; Malathion (1 µL, equivalent to 2.5 µg dry weight) was added as an internal standard.

The GC-MS analysis was performed on an Agilent 6890B GC oven coupled to a 5977B mass spectrometer (MS) detector (Agilent Technologies, Mulgrave, VIC, Australia) fitted with an MPS autosampler (Gerstel GmbH & Co.KG, Mülheim an der Ruhr, Germany). The GC oven was fitted with two 15 m HP-5MS columns (0.25 mm ID and 0.25 µm film thickness; 19091S-431 UI (Ultra Inert), Agilent Technologies, VIC, Australia) coupled to each other through a purged ultimate union (PUU) for the use of post-run backflushing. The sample (1.0 µL) was introduced via a multimode inlet (MMI) operated in split mode (1:20). The column was maintained at 40 °C for 5 min, followed by an increase to 250 °C at a rate of 10 °C/min. This was followed by a second increment to 310 °C at a rate of 60 °C/min. The column was held at 310 °C for 1 min. The mass spectrometer was kept in extractor ion mode (EI mode) at 70 eV. The GC-MS ion source temperature and transfer line were kept at 250 °C and 280 °C, respectively. Detector voltage was kept at 1054 V. The MS detector was turned off for the first 3 min and, at 4.0–4.8 min and 12.5–13.2-min time windows until the excess derivatization reagent (chloroformate/hexane solvents) were eluted from the column. This ensured that the source filament was not saturated and damaged. The scan range was kept in the range of *m*/*z* 35–350 (35–350 Daltons). Data acquisition and spectral analysis were

performed as described in a previous study (510) and qualitative identification of metabolites was performed according to the Metabolomics Standard Initiative (MSI) chemical analysis workgroup (511) using standard GC-MS reference metabolite libraries (NIST 17, and an in-house CF-based metabolomics library developed after Smart et al. (512) with the use of Kovats retention indices based on a reference n-alkane standard (C8-C40 Alkanes Calibration Standard, Sigma-Aldrich, Castle Hill, NSW, Australia).

4.3.12 Metabolic Phenotyping Analysis

Untargeted metabolomic profiling of faecal samples (n = 5 per group) were performed using GC-MS analysis by Dr. David J. Beale (CSIRO), Dr. Avinash V. Karpe (CSIRO) and Dr. Shakuntala V. Gondalia (Swinburne university of Technology) as described previously (510). Data analysis and interpretation was performed by the PhD candidate. The samples were subjected to derivatisation to increase volatility before subjecting to GC-MS analysis. Briefly, the samples (n = 5, weight = 40 mg) were freeze-dried and suspended in 1 mL methanol (LC-MS grade, Merck, Castle Hill, NSW, Australia), supplemented with 10 µg/mL adonitol (Analytical grade, Sigma Aldrich, Castle Hill, NSW, Australia) as an internal standard in a sterile 2 mL bead-beating tube. The samples were homogenized by bead beating for 30 s and then centrifuged at 570 g/4 °C for 15 min. The supernatant (50 µL) was transferred to a fresh centrifuge tube (1.5 mL) and dried in a vacuum evaporator centrifuge (LabGear, Brisbane, QLD, Australia) at 35 °C. Methoxyamine-HCl (20 mg/mL in Pyridine) (both, Analytical grade, Sigma Aldrich, Castle Hill, NSW, Australia) was added (40 µL) and samples were incubated at 30 °C/ 1400 rpm (ThermoMixer C, Eppendorf, Hamburg, Germany) for 90 min. This was followed by sialylation with 70 µL BSTFA at 37 °C/1400 rpm for 30 min. Pre-derivatized 13C-stearic acid (10 µg/mL) was added (1 µL) as the QA/QC internal standard. The mixture was briefly vortexed and centrifuged at 15,700 g for 5 min. The aliquot was transferred to vials for GC-MS analysis.

The GC-MS analysis was performed on an Agilent 6890B gas chromatograph (GC) oven coupled to a 5977B mass spectrometer (MS) detector (Agilent Technologies, Mulgrave, VIC, Australia) fitted with an MPS autosampler (Gerstel GmbH & Co. KG, Deutschland, Germany). The GC-MS conditions were as stated previously (513-515). Data acquisition and spectral analysis were performed using the Qualitative Analysis software (Version B.08.00) of MassHunter Workstation (Agilent Technologies). Qualitative identification of the compounds was performed according to the Metabolomics Standard Initiative (MSI) chemical analysis

workgroup (511) using standard GC-MS reference metabolite libraries (NIST 17, Fiehn Metabolomics RTL Library (G166766A, Agilent Technologies) and the Golm database) and with the use of Kovats retention indices based on a reference n-alkane standard (C8-C40 Alkanes Calibration Standard, Sigma-Aldrich, Castle Hill, NSW, Australia). For peak integration, a 5-point detection filtering (default settings) was set with a start threshold of 0.2 and a stop threshold of 0.0 for 10 scans per sample. Procedural blanks (n = 7) were analysed randomly throughout the sequence batch. The obtained data was processed on the Quantitative Analysis software of MassHunter Workstation and exported as a Microsoft Excel output file for statistical analysis.

GC-MS data imported to Microsoft Excel platform was normalized with respect to the internal standard adonitol (relative standard deviation = 11.257%). The normalized data was further log-transformed and auto-scaled (mean-centred) before statistical analysis (516). For analysis of metabolome variations, partial least squares-discriminant analysis (PLS-DA) and orthogonal (O) PLS-DA were used. Because PLS-DA can overfit data, we used 1000 permutations to validate these models. The OPLS-DA was used to identify discrimination between metabolites contributing to classification as previously described (510).

4.3.13 Statistical Analysis

The samples in the study were randomly chosen for all the analysis order to avoid bias. All data are presented as means ± standard error of the mean (SEM). The statistical analysis was performed with the use of GraphPad Prism Software (Version 7.0, San Diego, CA, USA). The data were evaluated using One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test to determine statistical differences between the groups against the DSS-control samples. For the analysis of DAI and body weight changes during the experimental period, two-way ANOVA followed by Tukey's post-hoc test was used, setting treatment and the time as the variables. A P-value of < 0.05 was considered significant. A MetaboAnalyst (Version 4.0, Wishart Research Group, University of Alberta) data annotation approach and Kyoto Encyclopaedia of Genes and Genomes (KEGG) Pathway Database were used for the hierarchical clustering analysis and significance analysis for microarrays (SAM), along with the variable importance of projection (VIP) (517). The SAM and VIP methods are well-established statistical methods for metabolites and were used to select the most discriminant and interesting biomarkers (518).

4.4 Results

4.4.1 Effects of B. coagulans, PSCF and Synbiotic supplementation on DAI and macroscopic inflammatory markers in DSS-induced mice

In comparison with the HC group, the administration of probiotic, prebiotic and synbiotic treatments in the respective groups did not show any sign of toxicity, which was evaluated by body weight increase, food intake and general appearance of the animals. DAI (cumulative score for body weight change, stool consistency and blood in faeces) was evaluated to determine the efficacy of the treatments in reducing the severity of disease symptoms in DSS-induced colitis (Figure 4.2A). Compared with the DSS-control group that showed severe colitis symptoms, pre-conditioning with *B. coagulans*, PSCF and Synbiotic combination significantly reduced the DAI levels as early as day 2 until the end of experiment. At the end of the experiment, DAI of DSS-control group was significantly high (5.8 ± 0.5) ($p \leq 0.0001$) compared with that of *B. coagulans* (3.1 ± 0.6, 47% reduction), PSCF (2.7 ± 0.5, 53% reduction), and Synbiotic (1.6 ± 0.2, 72% reduction) groups. DSS induction in DSS-control mice resulted in significant body weight loss until the end of experiment (−4.13 ± 1.4%). In contrast, mice maintained healthy body weight gain with *Bacillus* (2.84 ± 1.7%), PSCF (4.25 ± 1.0%), and synbiotic (4.7 ± 0.7%) treatments. Interestingly, PSCF was the more effective in reducing DAI starting from day 2 of DSS and in remediating the body weight loss as early as day 5 owing mainly to the impact of improvement in stool consistency on the DAI rating.

The macroscopic evaluation of colonic segments determined the beneficial effects of all three treatments used in our study as evidenced by substantial reduction in colon weight/body weight ratio (*B. coagulans*, 7.68 ± 0.2; PSCF, 9.24 ± 0.3 and Synbiotic, 8.74 ± 0.3 mg/g) compared with DSS-control group (11.12 ± 0.3 mg/gm) (Figure 4.2C). Intestinal inflammation is associated with spleen enlargement (499) and, as expected, relative spleen weight of untreated DSS-control mice was significantly higher (0.076 ± 0.004 g) than that of HC mice (0.054 ± 0.003 g). PSCF (0.062 ± 0.003 g) and Synbiotic (0.063 ± 0.002 g) were equally significantly effective in reducing spleen weight while *B. coagulans* did not affect the relative spleen weight (Figure 4.2D). In contrast to shortening of colon length (Figure 4.2E, 4.2F) in the DSS-control group (6.75 ± 0.3 cm), Synbiotic treatment proved effective in reducing this outcome by maintaining the colon length (8.12 ± 0.2 cm), which was significantly equal ($P = 0.99$) to that of the HC group (8.01 ± 0.2 cm). These markers are

considered to be directly correlated to the severity of colonic damage in this experimental model of colitis (499).

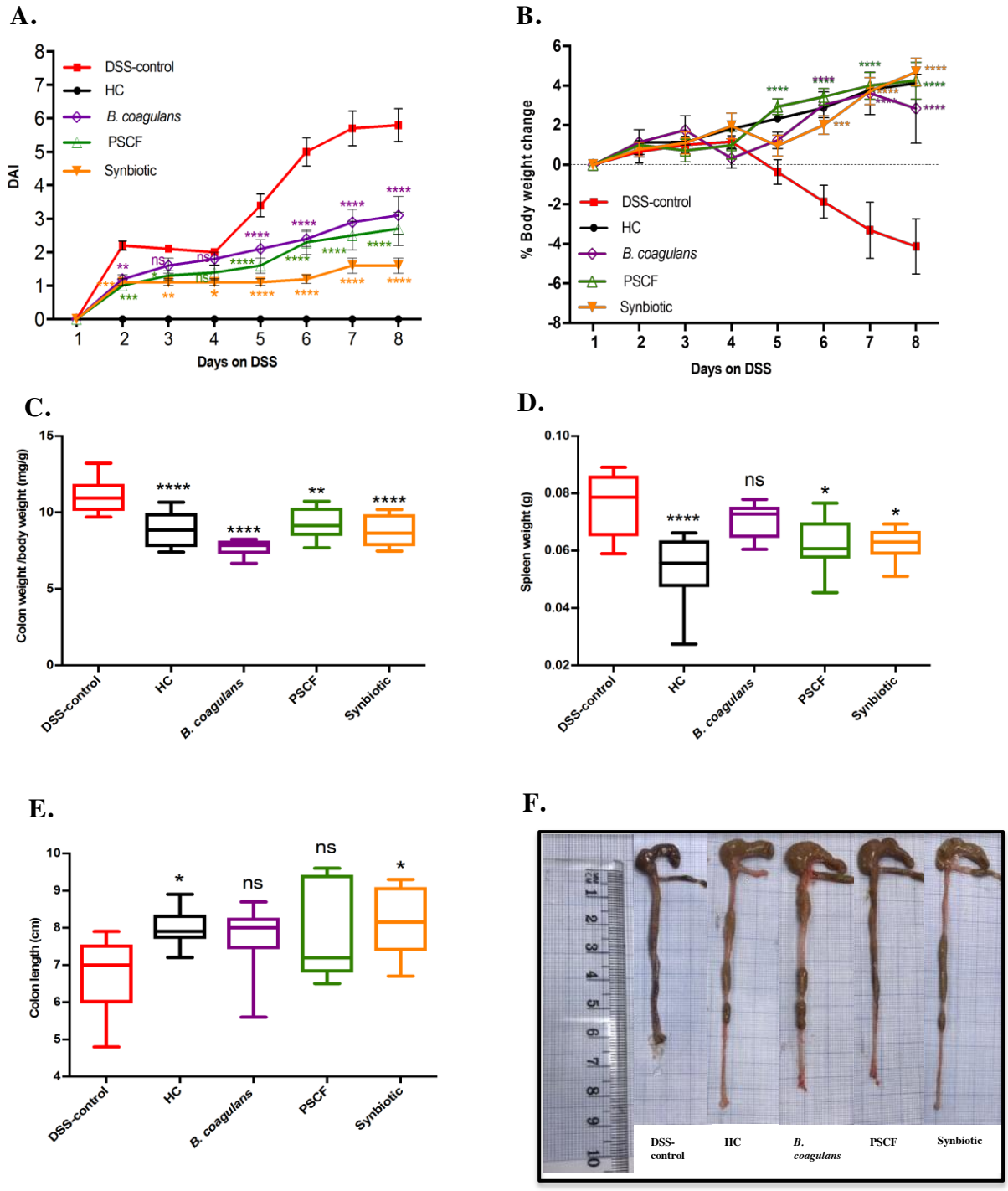


Figure 4.2. Effect of *B. coagulans* spores, PSCF and Synbiotic in DSS-induced colitis model. (**A**) Disease Activity Index (DAI), (**B**) % body weight change. Statistical significance among groups evaluated by two-way repeated-measures analysis of variance (ANOVA) followed by Tukey's test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 vs. DSS-control group and data expressed as mean ± SEM (*n* =10 per group). Colon weight/body weight ratio (**C**), Spleen weight (**D**), Colon length (**E**) and Macroscopic appearance of colon (**F**). Data expressed as mean ± SEM (*n* =10 per group), evaluated by one-way ANOVA followed by Tukey's Test. ns- non-significant, HC- Healthy control, PSCF- Prebiotic sugar cane fibre.

4.4.2 Effects of B. coagulans, PSCF and Synbiotic supplementation on histological alterations in DSS-induced mice

Histological (H&E staining) examination of proximal colon (PC) and distal colon (DC) sections of DSS-induced mice showed altered erosion or destruction of epithelium, crypt distortion, depletion of goblet cells, submucosal oedema, and inflammatory cellular infiltration in the colon, mostly affecting the DC (Figure 4.3A). HC mice showed no signs of histological colon damage with score 0, while DSS induction in DSS-control mice resulted in a cumulative damage score of 10.5 ± 0.8 for the PC and 17.4 ± 0.5 for the DC (Figure 4.3B). Supplementation of DSS-induced mice with *B. coagulans*, PSCF and Synbiotic treatments induced protection and repair of the colonic mucosa. *B. coagulans* and Synbiotic in particular, were more effective in retention of colonic structure, protection of crypts and goblet cells and rescued infiltration of inflammatory cells. This resulted in a significant overall reduction of cumulative histological score of DC (10.1 ± 1.2 and 7.38 ± 0.7 respectively). Relatively, PSCF also provided partial significant protection with histological score of 13.0 ± 1.0. In contrast, PCSF had no effect in PC (10.1 ± 0.5) with only *B. coagulans* and Synbiotic treatments being significantly successful in reducing damage to the PC with histological scores of 9.5 ± 0.7 and 7.8 ± 0.3 respectively. Unlike the DSS-control group, there was reduced polymorphic inflammatory infiltrate in the lamina propria and submucosa in probiotic, prebiotic and synbiotic supplemented group. This observation corroborates with the significantly reduced MPO activity in the colon of all three treatments (Figure 4.3C) compared with the DSS-control group. The attenuation of colonic inflammation in pre-conditioned mice (*B. coagulans*, PSCF and Synbiotic) is probably due to the anti-inflammatory properties of the functional dietary ingredients tested in this study.

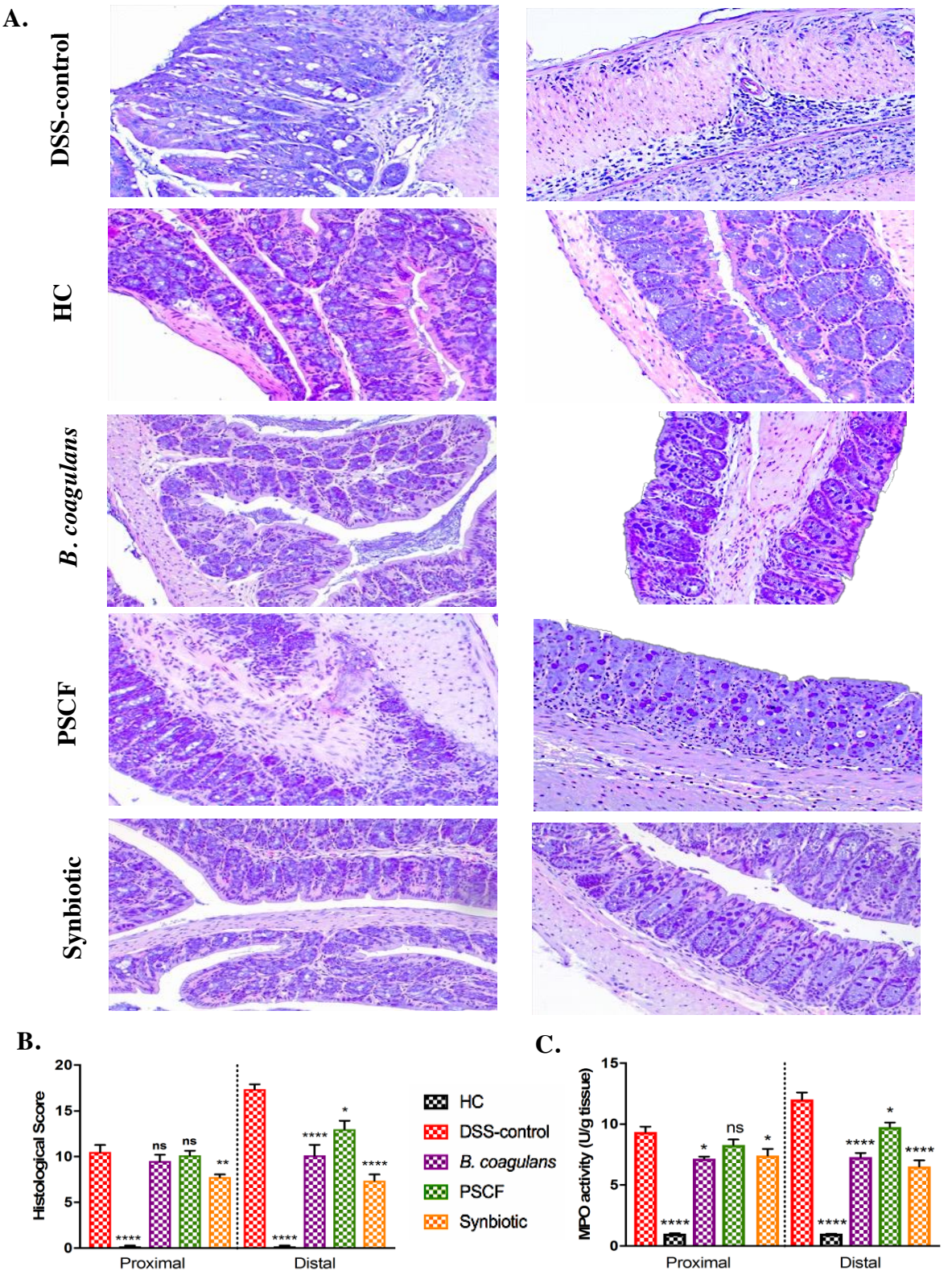


Figure 4.3. Effect of *B. coagulans* spores, PSCF and Synbiotic treatments on DSS-induced colon injury and inflammation. (**A**) Histological images of proximal and distal colonic tissues stained with hematoxylin and eosin at 20× for each experimental group. (**B**) Histological score calculated after microscopic analyses of proximal and distal sections of the colon. (**C**) Myeloperoxidase (MPO) activity in colonic tissues was determined by colorimetric assay. Results expressed as mean ± SEM (n = 8 per group), evaluated by one-way ANOVA followed by Tukey's test (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).

4.4.3 Effects of B. coagulans, PSCF and Synbiotic supplementation on goblet cells and colonic tight junction barrier

Histological examination of distal colon from DSS mice showed a depletion of goblet cells when compared to HC and pre-conditioned mice. This suggested beneficial effects of probiotic and prebiotic ingredients on the intestinal epithelium potentially through stimulating mucus secretion by goblet cells. Specific staining with Alcian blue was therefore carried out to assess the mucus production following the administration of probiotic, prebiotic and synbiotic treatments. As depicted in Figure 4.4, in comparison with DSS-control group, there was a higher level of mucus staining with Alcian blue in supplemented mice samples. This implied that there had been an induction of higher levels of mucus secretion in the DSS-challenged mice that received *B. coagulans* spores, Synbiotic, and PSCF supplementations. Unlike the DSS-controlled samples, where goblet cells were almost entirely destroyed, the mice supplemented with Synbiotic and *B. coagulans* showed protection of the goblet cells. PSCF also partially protected the goblet cells with mucus staining compared with DSS-control.

Immunohistochemical analysis was then performed to evaluate the assembly of the TJs and the integrity of the intestinal barrier. The presence of the TJ proteins-ZO-1, occludin, and claudin-1 on the tissue sections were analysed (Figure 4.5). In HC, ZO-1 (Figure 4.5A) staining was more intense in the apical tight junction complex both at the surface and in the crypts. In addition to showing their presence at the crypt surface, occludin (Figure 4.5B), and claudin-1 (Figure 4.5C) proteins stained more strongly at the basolateral membrane of crypts. As previously reported (519, 520) such signals were weak or totally absent on the epithelium of DSS-control sections resulting in very low percentage TJ protein expressions. Basolateral and partial apical staining of ZO-1, occludin and claudin-1 was maintained with *B. coagulans* and Synbiotic supplementation in DSS-treated animals. While PSCF was able to partially maintain ZO-1 and claudin-1 staining, such an effect was less evident for occludin. Synbiotic treatment was most effective in preserving the TJ protein expressions in DSS-induced mice further confirming its beneficial effects on the intestinal integrity.

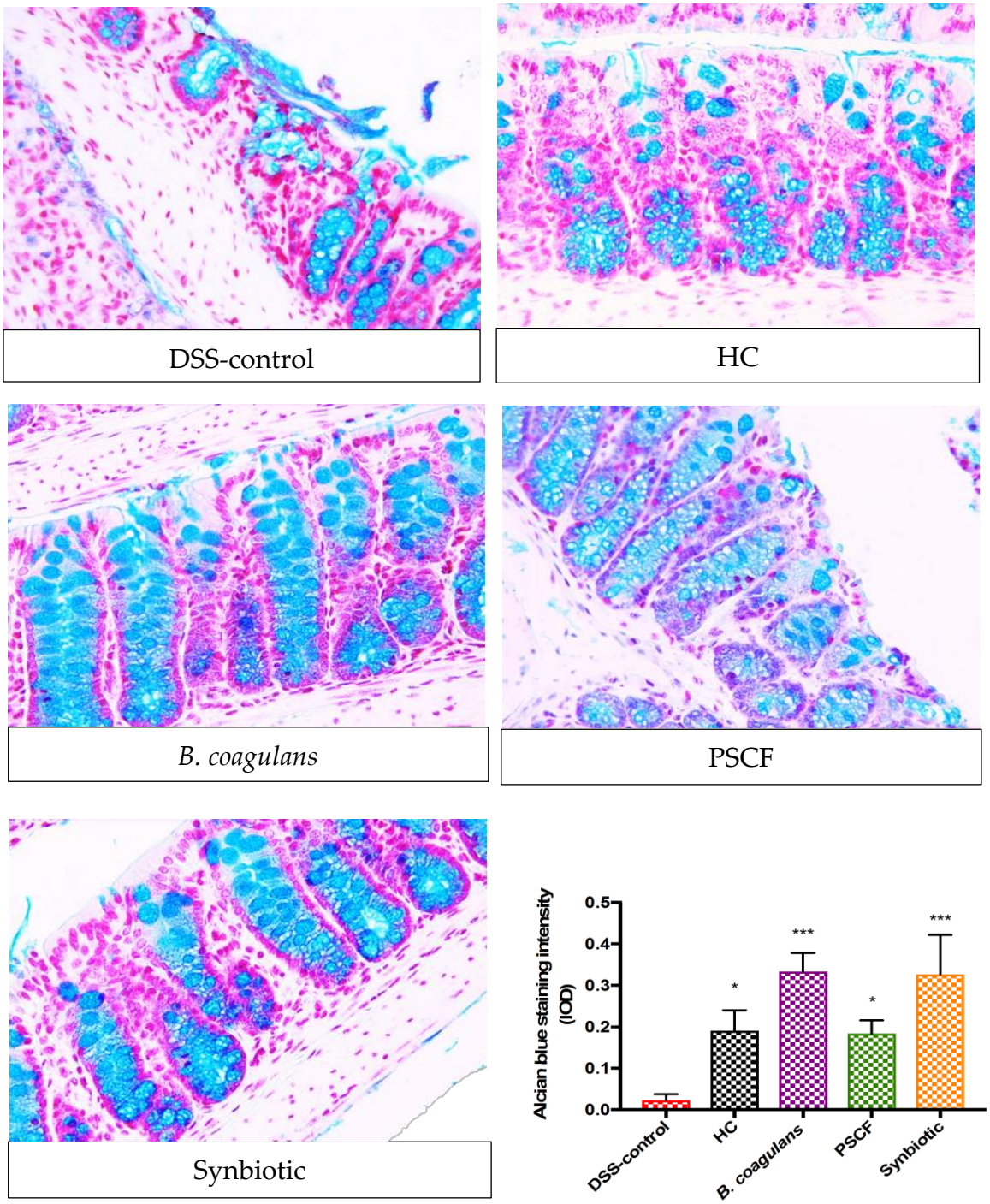


Figure 4.4. Effect of *B. coagulans* spores, PSCF and Synbiotic on goblet cells. The paraffin embedded sections were stained with Alcian Blue to detect changes in goblet cells and in production of mucus in distal colonic tissue in each experimental group (40×) and staining intensity (IOD) of respective group is illustrated in the graph. Results expressed as mean ± SEM (n = 4 per group), evaluated by one-way ANOVA followed by Tukey's test (*$P < 0.05$, ***$P < 0.001$).

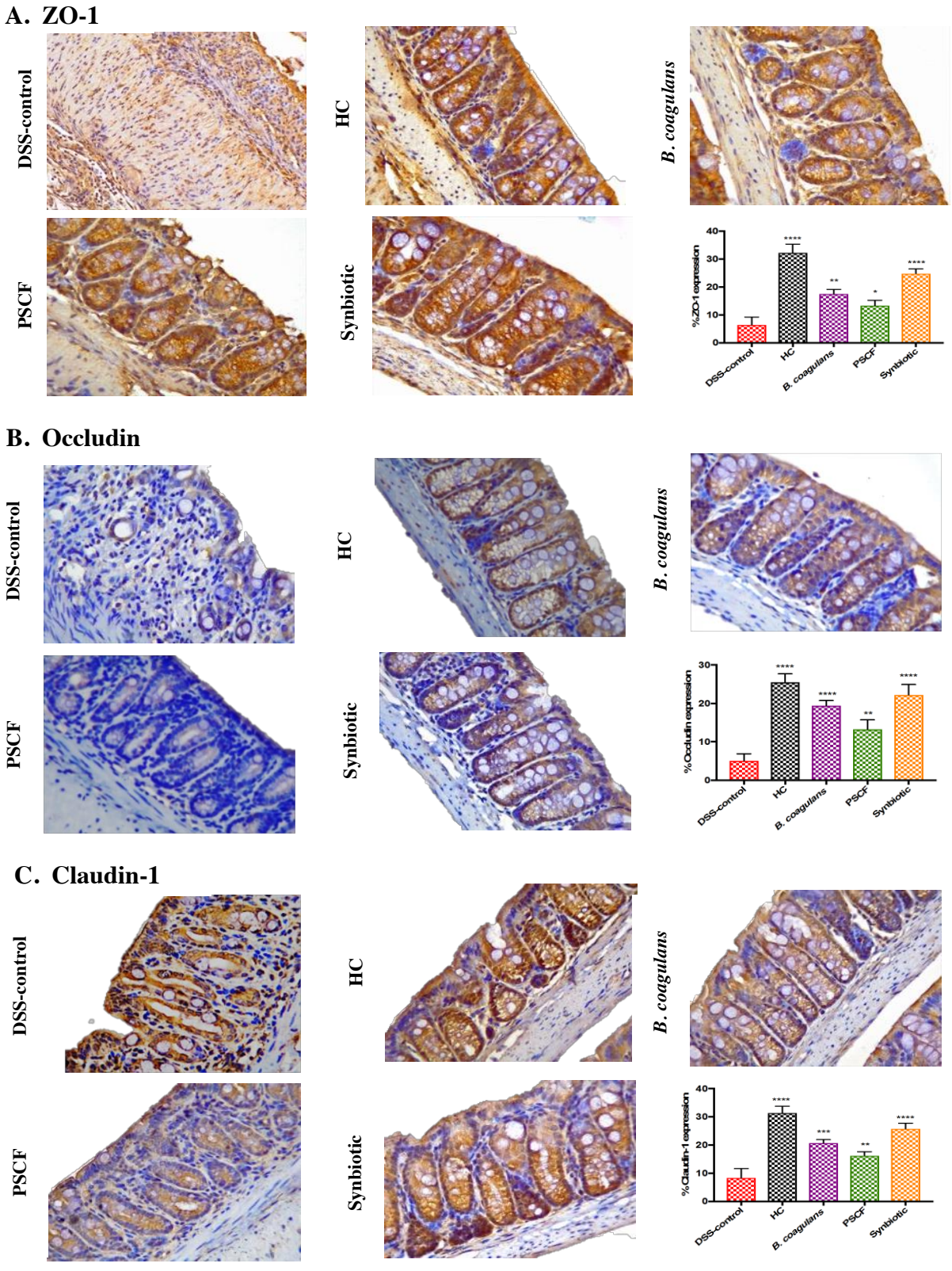


Figure 4.5 Effects of *B. coagulans* spores, PSCF and Synbiotic on expression of epithelial tight junction proteins. Immunohistochemical detection of (**A**) ZO-1, (**B**) Occludin and (**C**) claudin-1 and its respective percentage of expression in colon at 40×. Data expressed as mean ± SEM (n = 4 per group) and statistical significance among groups evaluated by one-way ANOVA followed by Tukey's test *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs. DSS-control group.

4.4.4 Immunomodulatory effects of B. coagulans, PSCF, and Synbiotic supplementation on immune markers in DSS-induced mice

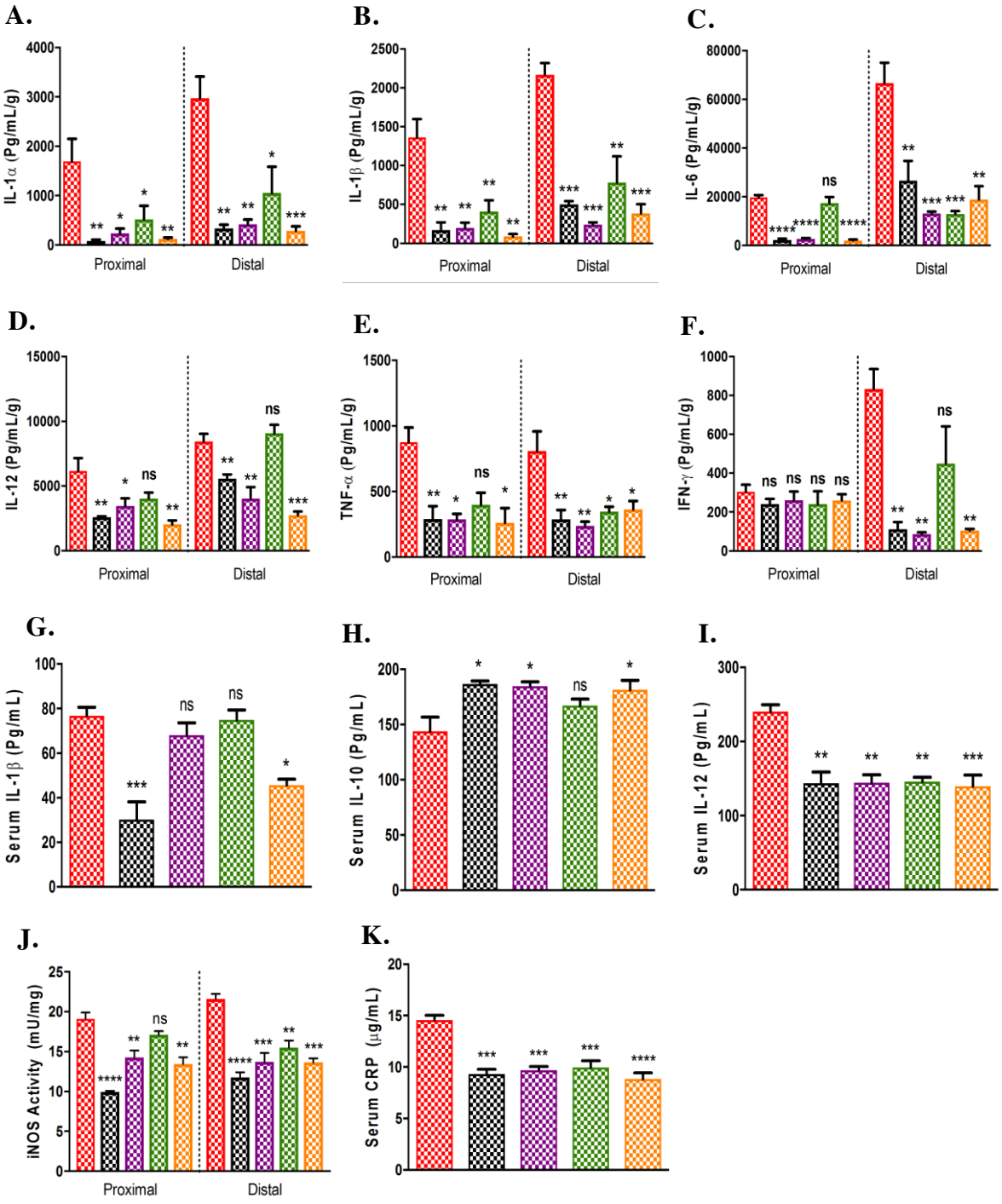


Figure 4.6. Effect of *B. coagulans* spores, PSCF and Synbiotic on immune markers in colon tissues and blood serum. Protein levels of cytokines including (**A**) IL-1α, (**B**) IL-1β, (**C**) IL-6, (**D**) IL-12, (**E**) TNF-α, (**F**) IFN-γ in proximal and distal colon explants as well as cytokine levels of (**G**) IL-1β, (**H**) IL-10, and (**I**) IL-12 in blood serum were analysed by Bio-plex. iNOS activity in colon tissues (**J**) measured by NOS activity assay and CRP levels in serum (**K**) by ELISA. Statistical significance among groups evaluated by one-way ANOVA followed by Tukey's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs. DSS-colitic group and data expressed as mean ± SEM ($n = 3$ per group).

The cytokine analysis of the colonic segments and serum measured the intestinal immunomodulatory and anti-inflammatory effects of *B. coagulans*, PSCF and their synbiotic combination. A beneficial impact of ameliorating the altered immune response induced by DSS intake was noted. Overall, probiotic, prebiotic, and synbiotic treatments remarkably reduced the pro-inflammatory cytokine secretions of IL-1α, IL-1β, IL-6, IL-12, TNF-α, and IFN-γ in proximal and distal colon segments compared with that of the DSS-colitic segments (Figure 4.6), while no significant effect of supplementations was noted on other cytokine levels (Supplementary Figure SF4.1). *B. coagulans* and Synbiotic treatments were equally effective in maintaining the levels of these altered cytokines relative to that of non-colitic mice while PSCF did not show significant effective reduction of IL-12 cytokine levels (Figure 4.6D). Synbiotic treatment was statistically more potent in suppressing the elevated levels of IL-1α (−90.29%) and IL-12 (−67.42%) compared with *B. coagulans* (−85.94%, −52.20%, respectively) in the DC. No positive effect was observed in the PC segment for IL-6 and TNF-α levels by PSCF treatment. However, the excellent immunomodulatory effect in the DC for respective cytokines was confirmed (Figure 4.6C, 4.6E), implicating its differential effects in these colonic segments.

Serum cytokines IL-1β, IL-12, and IL-10 also showed immunomodulatory effects (Figure 4.6G–I), but no significant effect was observed for other serum cytokines (Supplementary Figure SF4.1). All three treatments substantially restored the IL-12 levels to values similar to HC mice, while Synbiotic treatment was significantly more efficacious in reducing pro-inflammatory IL-1β levels in serum. Moreover, *B. coagulans* and Synbiotic treatments increased the anti-inflammatory IL-10 levels in serum (181.2 ± 8.70, 184.7 ± 3.81 pg/mL respectively) compared with the DSS-colitic group (143.8 ± 12.80 pg/mL) (Figure 4.6H). iNOS activity which is known to be high in colitis in response to pro-inflammatory stimuli (508), was significantly suppressed in both colon segments by all treatments (Figure 4.6J) compared with DSS-colitic levels. Serum CRP level (Figure 4.6K) was significantly higher in the DSS-control group (14.58 ± 0.45 μg/mL) in comparison with HC animals (9.32 ± 0.45 μg/mL). *Bacillus*, PSCF and Synbiotic remarkably normalised the CRP levels (9.67 ± 0.34, 9.95 ± 0.65, 8.83 ± 0.59 μg/mL, respectively) to that of the HC group. These results confirmed the notable anti-inflammatory efficacy of probiotic *Bacillus* spores, prebiotic PSCF and their synbiotic combination used in the study.

4.4.5 Effects of B. coagulans, PSCF, and Synbiotic supplementation on altered faecal metabolic profile in DSS-induced mice

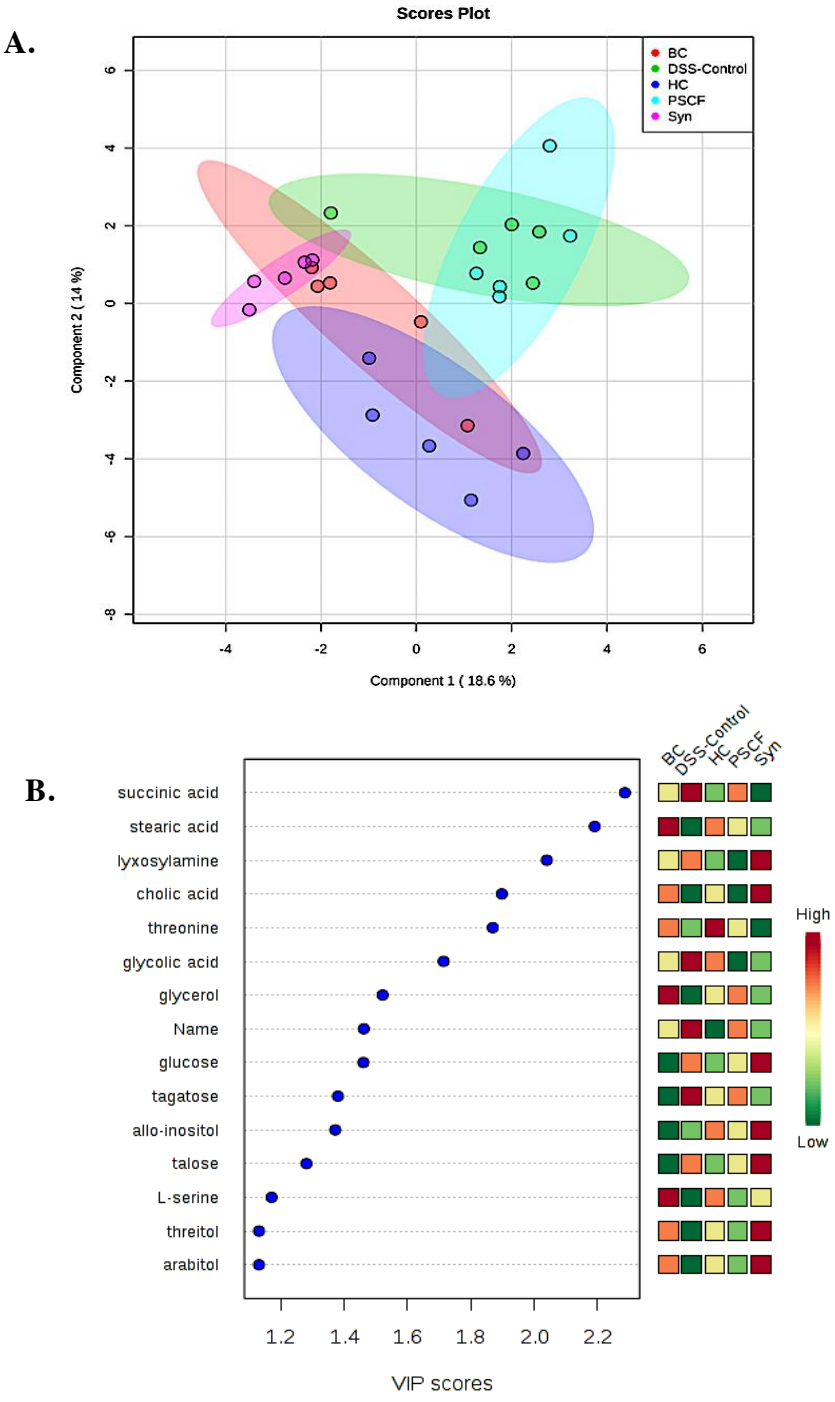


Figure 4.7. Effect of *B. coagulans* spores, PSCF and Synbiotic on metabolic modulations in DSS-induced colitic mice. (**A**) 2D-PLS-DA plot showing spatial division among groups that received different supplementations, DSS-control mice that received no supplementation and HC. (**B**) Key compounds separating the groups based on variable importance projection (VIP) score plot in PLS-DA analysis. (BC-*B. coagulans*, Syn-synbiotic).

Faecal metabolites were analysed using GC-MS to gain an overview of changes induced by *B. coagulans*, PSCF and Synbiotic supplementation in DSS-treated mice. A total of 61 metabolites of different functional groups such as sugars, amino acids, volatile fatty acids, and biogenic amines were detected. A supervised partial least squares-discriminant analysis (PLS-DA) was performed to evaluate metabolic phenotyping of each experimental group as shown in Figure 4.7A. The samples from HC and DSS-control clusters were clearly divergent indicating marked distinction in metabolic patterns between the two groups. While, clusters of supplemented mice samples overlapped with that of HC and DSS-control, clear demarcation of the synbiotic and *B. coagulans* cluster was noted, with PSCF showing only partial divergence relative to that of DSS-control. This indicates substantial efficacy of *B. coagulans* to metabolise PSCF that, in turn, induced significant biochemical changes potentially owing to their synergistic effects as evidenced from synbiotic samples. Combination of PLS-DA (R^2Y= 0.810 (P = 0.01), Q^2 = 0.710), VIP scores (Figure 4.7B) and significance analysis for microarrays (SAM) enabled us to identify potential biomarkers. The results showed 61 metabolites with 40 statistically significant compounds contributing to the clustering, with their SAM scores fold changes and International Chemical Identifiers (InChI) and standard InChI hashes (InChIKey IDs) listed in Supplementary Table ST4.1. Key metabolic markers making a significant contribution were identified by VIP analysis as displayed in Figure 4.7B. Among these identified metabolites, noticeable differences between DSS-control, and HC samples were noted particularly for succinic acid, stearic acid, and glycerol. Synbiotic supplementation was beneficial in minimising the metabolite alterations induced by DSS (Figure 4.7B and Supplementary Table ST4.1).

4.4.6 Effects of B. coagulans, PSCF and Synbiotic supplementation on SCFA levels in DSS-induced mice

As shown in Figure 4.8, supplementation of DSS-induced mice with *B. coagulans*, PSCF, and Synbiotic treatments induced substantial modulations in the SCFA concentrations and their effects varied across caecal, luminal and faecal contents. Overall, the highest concentration of SCFA were noted in caecal contents compared to mucosal-associated and faecal contents. There were no significant differences between the concentration of acetate and propionate in DSS-control and HC mice. PSCF however, induced a significant increase in the acetate levels in caecal and mucosal-associated samples while, Synbiotic treatment was most significant in elevating acetate concentrations in mucosal-associated samples. All three

supplementations were significantly effective in improving the propionate concentrations in caecal and mucosal-associated contents while, only synbiotic supplementation increased propionate levels in faecal samples.

Butyrate levels were significantly decreased by DSS administration (2.05 ± 0.6 μg/g) compared with that of HC mice (7.91 ± 1.1 μg/g) in the caecum. The decrease in butyrate induced by DSS was maintained at control levels with all three supplements in the caecum with PSCF (13.2 ± 2.4 μg/g) being significantly more effective than *B. coagulans* (11 ± 0.9 μg/g) and Synbiotic (10.4 ± 1.0 μg/g). Similarly, in faecal content, PSCF significantly improved butyrate (13.6 ± 1.6 μg/g compared with 3.72 ± 0.1 μg/g for DSS-colitic followed by Synbiotic (8.15 ± 0.6 μg/g) while *B. coagulans* (6.69 ± 0.1 μg/g) had no effect. Synbiotic supplementation resulted in marked increase in mucosal-associated valerate while, PSCF caused elevation in its concentration in faecal content. Similarly, Synbiotic was more efficient in reducing elevated succinate levels.

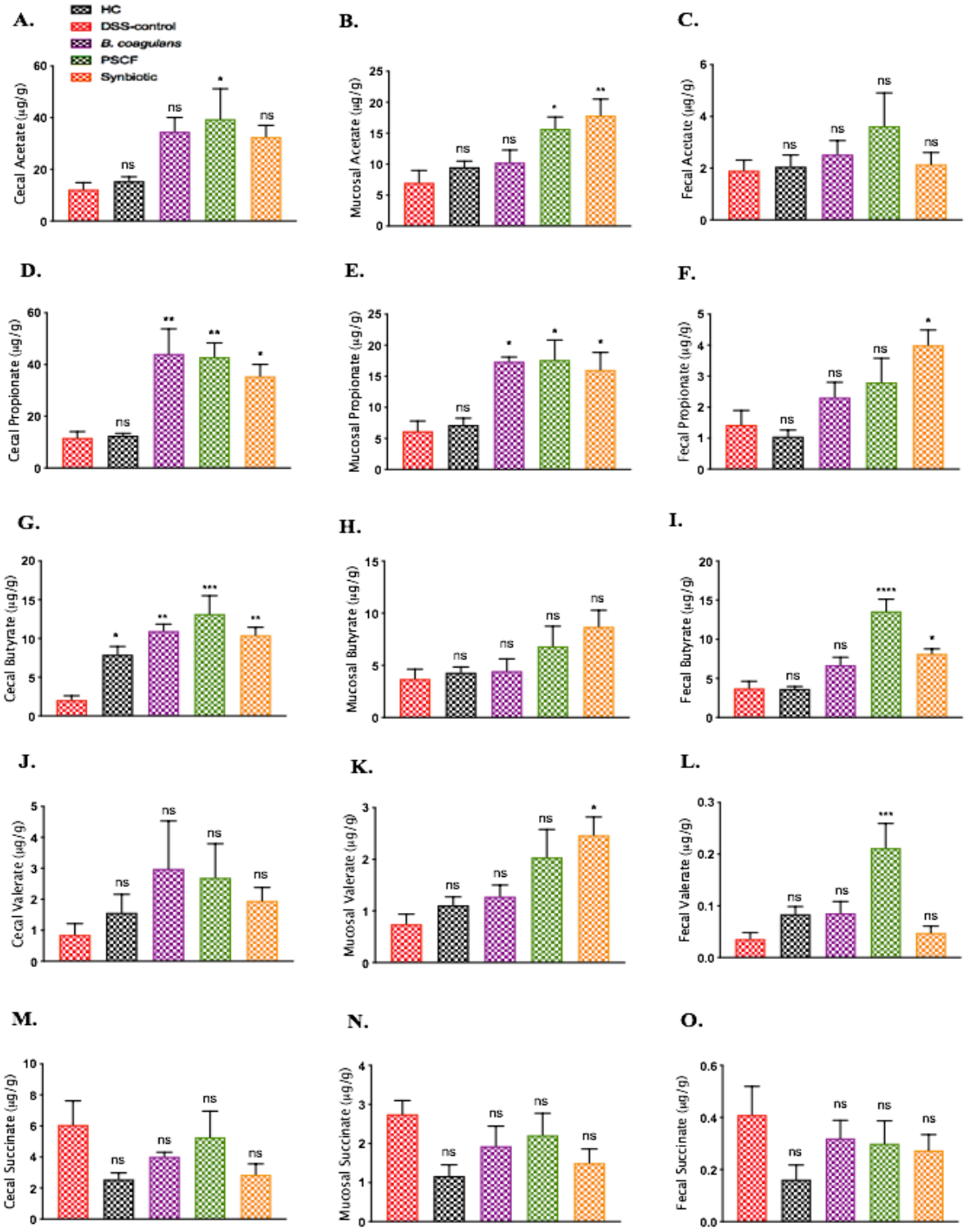


Figure 4.8. Effects of *B. coagulans* spores, PSCF and Synbiotic in modulating SCFA concentrations in caecal, mucosal-associated and faecal contents in DSS-induced colitis. Caecal- acetate (**A**), propionate (**D**), butyrate (**G**), valerate (**J**), succinate (**M**); mucosal-associated acetate (**B**), propionate (**E**), butyrate (**H**), valerate (**K**), succinate (**N**) and faecal- acetate (**C**), propionate (F), butyrate (**I**), valerate (**L**), succinate (**O**). Statistical significance among groups evaluated by one-way ANOVA followed by Tukey's test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 vs. DSS-colitic group and data expressed as mean ± SEM (*n* = 5 per group). ns-non-significant.

4.5 Discussion

Dietary strategies involving probiotic and prebiotic fibre components that function by modulating immune responses, colonic epithelial integrity and microbial composition and related metabolites are being widely investigated for the prevention or reduction of severity of IBD (521, 522). The present study clearly supported the premise that conditioning of the gut with synbiotic supplementation containing compatible probiotic and prebiotic fibre can be greatly beneficial to reducing the symptoms and severity of DSS-induced acute colitis in mice. The observations confirmed substantial anti-inflammatory efficacy by synbiotic supplementation containing *B. coagulans* and PSCF. This was evidenced by the improvement of clinical symptoms, macroscopic, histological, biochemical, metabolic and immune parameters in the DSS-induced colitic mice model.

The addition of 2% (*w*/*v*) DSS to drinking water for seven days without ameliorating treatments resulted in a progressive rise in DAI (Figure 4.2A), owing to both body weight loss (Figure 4.2B) and excretion of diarrheic/bleeding faeces. However, the supplementation of DSS-induced mice with *B. coagulans*, PSCF, and their synbiotic combination significantly attenuated the severity of the DSS damage and improved DAI and macroscopic markers of inflammation (Figure 4.2C–F). The ability of PSCF to show early effects on DAI and body weight could be related to its high content of insoluble hemicellulose. This fraction has a large water-holding capacity and thus could appropriately contribute to regulating the faecal water content in colitic mice (288, 289). The anti-diarrheal effect of *B. coagulans* has been previously confirmed (48). The increased beneficial effects of synbiotic supplementation in reducing the disease severity could be related to the synergistic actions between the probiotic and prebiotic components.

A potentiated synbiotic effect relative to that of *B. coagulans* and PSCF individually was also evident from the histology of the colon compared with DSS-control mice (Figure 4.3A,B). Synbiotic supplementation showed substantial protection to the colonic epithelial architecture by mitigating crypt disruption, loss of goblet cells, submucosal oedema and epithelial structure damage induced by DSS. Synbiotic supplementation also induced suppression of infiltration of activated neutrophils as evidenced by significant reduction in MPO activity in DSS-induced mice (Figure 4.3C). The infiltration of activated neutrophils is one of the most prominent histological features observed in IBD and is directly proportional to the MPO activity. Superoxide anions and other reactive species produced by neutrophils

leads to tissue necrosis and mucosal dysfunction in IBD (520). The reduction in MPO activity suggests that synbiotic supplementation imparted an anti-inflammatory effect, in addition to histological protection.

Disruption of intestinal epithelial TJs and impaired epithelial barrier function is a central event in the pathogenesis of IBD and may lead to persistent aberrant immune reactions, thus accelerating gut inflammation and the inflammatory circuit (99). Synbiotic treatment in our study was the most effective followed by *B. coagulans* and PSCF in protecting the expression level of TJ proteins (ZO-1, occludin and claudin-1) in DSS-induced mice (Figure 4.5). TJs create a semi-permeable barrier against paracellular penetration of harmful substances from the gut lumen (99). *Bacillus subtilis* intake has been recently confirmed to upregulate the expressions of TJ proteins for improved barrier function in colitic mice (392) and corroborates with these results. Moreover, Synbiotic and *B. coagulans* protected the goblet cells and mucin production more effectively than PSCF alone (Figure 4.4). This indicates the possible ability of the *B. coagulans* MTCC 5856 spores to benefit goblet cell structure and function, and needs further investigation to determine the mechanism of this effect. Some *Bacillus* species have been shown to upregulate mucin glycoproteins and protect colonic mucus layer integrity and goblet cell function (523). Although we could not confirm if Synbiotic supplementation had a stimulating effect on TJ proteins and/or localisation or, instead, if it avoided TJ degradation by DSS, we observed that Synbiotic supplementation significantly maintained the TJ patterns similar to that of animals in HC. This is indicative that Synbiotic supplementation significantly preserved the integrity of the epithelium. The synergy between *B. coagulans* and PSCF could have imparted excellent protection and/or maintenance of epithelial integrity on DSS-induced mice, thus supporting its application in IBD to reinforce intestinal barrier integrity.

Alterations in the barrier integrity in IBD leads to aberrant immune responses resulting in an inflammation cascade and tissue damage (91). Although *B. coagulans* and PSCF supplementations alone were able to modulate the tested cytokines (IL-1α, IL-1β, IL-6, IL-12, TNF-α, and IFN-γ), a more profound anti-inflammatory effect was observed with Synbiotic supplementation in both the colon and serum (Figure 4.6A–I). A spike in the levels of IL-1β, IL-6, and TNF-α have been implicated in human IBD pathogenesis (118). Such pro-inflammatory cytokines are secreted at high levels by activated lamina propria antigen presenting cells in response to inflammation. Pro-inflammatory cytokines such as IL-6, TNF-

α, and IL-1β are being targeted by therapeutic approaches to curb the aberrant inflammatory response in IBD due to their roles in the pathogenesis of the disease (524-526). TNF-α is reported to exert its pro-inflammatory effect through elevated production of IL-6 and IL-1β (527). This is in line with the present study, where DSS-induction caused elevated secretions of these cytokines in DSS-control group relative to that of HC (Figure 4.6). Upregulation of pro-inflammatory cytokine levels has also been reported to upregulate iNOS expression and secretion of nitric oxide that causes tissue damage in IBD (528). The anti-inflammatory effects of Synbiotic, *B. coagulans* and PSCF in suppressing the levels of these pro-inflammatory cytokines as well as reducing iNOS activity in colonic tissues, indicates their immunomodulatory potentials. *B. coagulans* MTCC 5856 spores in our previous study showed excellent immunomodulatory effect *in-vitro* (42). Marked reduction in pro-inflammatory cytokines in the colon of DSS-induced mice in the current study further supports its excellent immunomodulatory efficacy in IBD application.

Synbiotic supplementation was the most effective in demonstrating noticeable anti-inflammatory effects by significantly reducing the serum levels of pro-inflammatory IL-1β and IL-12 while concurrently elevating anti-inflammatory IL-10 in the serum. IL-10 plays a prominent role in counterbalancing Th1 and Th17 immune activity in IBD towards a Th2 response by downregulating antigen presentation and subsequent release of proinflammatory cytokines thereby attenuating mucosal inflammation (527). IL-10 has been reported to play a role in maintaining intestinal barrier integrity possibly owing to effects on zonulin pathway (529). The ability of *B. coagulans* spores to elicit IL-10 levels in inflammatory condition has been determined *in-vitro* and in human subjects. Thus, the anti-inflammatory efficacy of Synbiotic supplementation could be related to major immune-regulating capability of *B. coagulans* MTCC 5856 spores, thereby supporting its application in synbiotic therapies for IBD. Furthermore, Synbiotic treatment also reduced the increased CRP levels in the serum of DSS-induced mice. In the inflammatory state, circulating IL-6 promotes CRP production in the liver and its release into the bloodstream (530). Elevated levels of CRP has been implicated in human IBD patients (531). The reduction in overall pro-inflammatory mediators by synbiotic supplementation may be due to either direct immune-regulating effect of the *B. coagulans* and PSCF on cytokine secretion, or it could be owing to their indirect affect on the protection of intestinal barrier integrity. In either case this leads to reduction in luminal antigens and full activation of the innate immune system.

DNA extracted from caecal, faecal and mucosal-associated contents from DSS-induced mice in the present study, failed amplification before 16s rRNA sequencing. This was caused by the presence of DSS in the samples that inhibited the PCR amplification as known earlier (532). The microbiota profiling of DSS samples could therefore not be performed and is a limitation of the study. The levels of microbiota-derived untargeted metabolites and SCFAs were therefore alternatively analysed as signatures of the gut microbiota that contribute to modulating the immune activity of the intestinal mucosa (533, 534). The untreated DSS-control mice exhibited distinct faecal metabolic phenotype relative to that of HC. This was reflective of clinical and animal IBD studies that confirmed significant differences in metabolic profiles between healthy and IBD patients (217, 220, 221). The metabolic analysis of supplemented mice in our study resulted in considerable normalization of metabolic profile indicating the positive effects of synergistic combination of *B. coagulans* and PSCF that induced marked improvement in metabolic pattern. Notably, for mice supplemented with PSCF, there was not much distinction observed from DSS-control group, but the synbiotic combination with *B. coagulans* resulted in an improved metabolic pattern. This marked synergism could be associated with the acceleration of fermentation of insoluble plant cellular materials, such as hemicellulose in PSCF, by the supplemented *B. coagulans* (71). The resulting higher levels of fermentation metabolites would thus, in turn, influence other beneficial microbial metabolic activities. This further supports the application of compatible synbiotic components to generate maximum benefits through increased SCFA production.

Increased levels of microbiota-derived SCFAs are inversely associated with dysbiosis in IBD (229). Of particular interest are higher levels of acetate, propionate and butyrate, which results from fermentation of indigestible carbohydrate components of fibre-rich diets. The effects of SCFAs have been studied to in animal models of colitis (288) and clinical UC (235). Each type of SCFA is likely to contribute to host health (535). In this study we determined the SCFA profile along the GIT, analysing samples across caecal, mucosal-associated, and faecal contents (Figure 4.8). The concentration of SCFAs varied along the length of the gut, with most abundant levels in the caecum and PC, while it declined towards the DC (535). In line with a recent study (536), caecum showed the highest levels of all the SCFAs tested in our study irrespective of the supplementation. Caecum is considered the major site for fermentation in the rodent gut and contains the largest pool of microbiota. It therefore generates the most SCFAs. However, some recent studies have also reported changes in the microbiota and associated amounts of metabolites in along different regions of

GIT (215, 536, 537). Therefore, the overall gastrointestinal profile is of importance when associating the gut microbiome and metabolites with health outcomes.

All three supplementations caused a substantial increase in concentrations of measured SCFAs compares with that of DSS-control group. However, the potentiated effect of Synbiotic supplementation is evidenced by induction of SCFA production along the entire length of the colon compared to *B. coagulans* supplementation alone where the additional SCFA production capacity was absent past the caecum. This effect highlights the advantage of using a compatible prebiotic fibre as synbiotic companion with a particular probiotic that can metabolize it. The high total dietary fibre content of PSCF (87%) (75) would contribute to this effect. PSCF supplementation resulted in increased butyrate levels line with an in-vitro study with human gut microbiota utilizing sugar cane fibre (75).The effect of additional SCFA production in caecum by *B. coagulans* would not extend to the proximal or distal parts of the colon. In the current study, the elicited extra SCFAs with synbiotic supplementation from caecum to the faecal pellets, indicated the ability of *B. coagulans* to utilize the PSCF to also generate SCFAs after the caecum. The ability of *B. coagulans* to metabolize a variety of plant fibres for fermentation, including cranberry fibre (478) and fenugreek seeds (477), to produce SCFAs and hemicellulose (71) for lactic acid production has been previously demonstrated.

PSCF supplementation also resulted in increased butyrate levels, correlating with results of an *in-vitro* study with human gut microbiota utilizing sugar cane fibre (75). This ability of synbiotic supplementation for eliciting butyrate levels along the entire length of colon could contribute to the beneficial effect observed in the current study. Butyrate is the preferred energy source for colonocytes and has the ability to regulate cytokines thus showing protection against inflammation in UC and colorectal cancer (535). Butyrate has been demonstrated in *in-vitro* (538, 539) and *in-vivo* (540) studies to increase epithelial integrity and mucus secretion, consistent with the immunohistological and mucus staining analysis in the present study. The substantial increase in butyrate levels in the caecum by *B. coagulans* supplementation may be due to ability to support the growth and activity of butyrate producers probably via cross-feeding of the lactic acid production. *B. coagulans* are known to be efficient at producing lactic acid through fermentation of various plant substrates, including hemicellulose (64, 71). Lactic acid is reported to be utilized by strictly anaerobic butyrate-producing bacteria of clostridial clusters XIVa for the production of high concentrations of butyric acid (541). Thus, the synbiotic approach with probiotic bacteria and

prebiotic fibre that directly or indirectly influence butyrate production may help to restore intestinal barrier integrity in diseased state. This effect was evidenced by the significant reduction in DSS-induced colonic epithelial damage (Figure 4.3) by Synbiotic supplementation in our study.

Acetate and propionate have also been studied to benefit epithelial integrity via binding with certain metabolite-sensing G-protein-coupled receptors (such as GPR43, GPR109A) and modulating immune response (227, 239, 542). Valerate was increased by Synbiotic supplementation. It that has been determined to stimulate intestinal growth and attenuate inflammatory pathogenesis in colitis and cancer (222). Besides their positive effect in colon, SCFAs have also been exhibited to mediate improved host metabolism and modulate the activity of the enteric nervous system (535), thus providing benefits beyond GIT. The high levels of immuno-modulatory effects observed in the present study could also possibly be correlated to high SCFA levels induced by Synbiotic supplementation owing to the synergistic combination. SCFAs by engaging with engage with GPRs are known to induce immune-modulation leading to a direct local and systemic anti-inflammatory effects (227, 543). This further supports the application of synergistic synbiotic combinations to achieve maximum benefits in resolving the inflammatory circuit in IBD.

4.6 Conclusions

This is a detailed study highlighting the site-specific inflammatory and SCFA changes in a mice model of IBD as a result of synbiotic supplementation of the normal diet with prebiotic whole plant fibre and probiotic spores. The Synbiotic pre-supplementation resulted in a substantial anti-inflammatory effect, reducing disease severity, colonic damage, and inflammatory mediators while modulating the metabolite and SCFA profiles of DSS-induced gut damage. The research has clearly demonstrated that the supplementation of whole plant PSCF and *B. coagulans* spores produced a synergistic combination that protected mice against acute damage induced by DSS in mice. The results underscore the significant efficacy of synbiotic applications to increase the beneficial and preventive effects on the host by targeting different mechanistic approaches to resolving the inflammation cycle. However, the differences in the evolved biology of humans compared to mice requires caution in translation of the results to impacts on human disease (219). While mice models do allow the changes in gut microbiota, as a result of pre- and probiotic combinations, to be studied in a controlled experiment direct human trials will be needed. The delineation of the synergistic biological

actions of probiotic *B*. *coagulans* spores and prebiotic PSCF in mouse model of IBD provides support for investigating their therapeutic and preventive effects in human IBD. However, the ability to reduce the severity of DSS-induced colitis was demonstrated using pre-supplementation. Human trials should be aimed at testing proactive prevention, or efficacy after partial control of inflammatory disease, such as in association with drug treatment.

4.7 Supplementary data

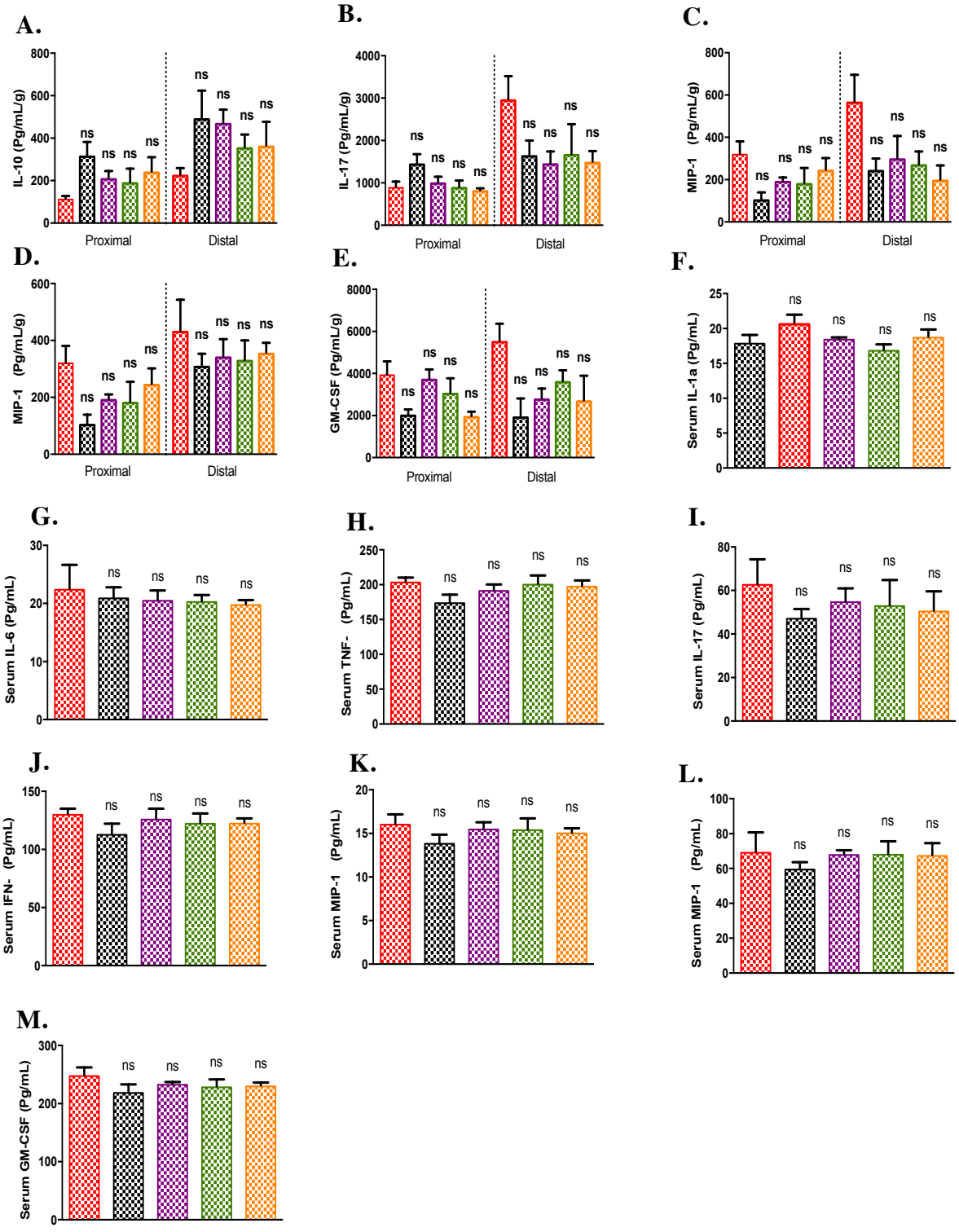


Figure SF4.1. Non-significant effect of *B. coagulans* spores, PSCF and Synbiotic on immune markers in colon tissues and blood serum. Protein levels of cytokines including (**A**) IL-10 (**B**) IL-17, (**C**) MIP-1α, (**D**) MIP-1β, (**E**) GM-CSF in proximal and distal colon explants as well as cytokine levels of (**F**) IL-1α, (**G**) IL-6, (**H**) TNF-α, (**I**) IL-17, (**J**) IFN-γ, (**K**) MIP-1α, (**L**) MIP-1β, (**M**) GM-CSF in blood serum were analysed by Bio-plex. Statistical significance among groups evaluated by one-way ANOVA followed by Tukey's test. Non-significant (ns) vs. DSS-colitic group and data expressed as mean ± SEM (n = 3 per group).

Table ST4.1 Most significant compounds identified by OPLS-DA and SAM analysis in HC, DSS-control, *B. coagulans* (BC), PSCF and synbiotic groups (*First 40 compounds are identified by SAM).

Compound name	InCHI Key	DSS-control (FC)	BC (FC)	HC (FC)	PSCF (FC)	Synbiotic (FC)	SAM (*P* value)
Oxalic acid*	KZSNJWFQEVHDMF-BYPYZUCNSA-N	1.3174	1.255	0.55868	1.1763	1.1165	0.0009836
Urea*	XSQUKJJJFZCRTK-UHFFFAOYSA-N	1.8432	1.6662	1.0792	1.6115	1.5227	0.000983
Allantoin*	POJWUDADGALRAB-UHFFFAOYSA-N	2.148	1.9001	0.68545	1.7891	1.704	0.002950
Stearic acid*	QIQXTHQIDYTFRH-UHFFFAOYSA-N	1.8728	1.7465	2.1912	1.6452	1.5837	0.0031148
Lyxosylamine*	RQBSUMJKSOSGJJ-AGQMPKSLSA-N	1.582	1.4033	2.0398	1.3617	1.3011	0.0057377
L-proline*	ONIBWKKTOPOVIA-BYPYZUCNSA-N	0.15132	0.33928	0.11327	0.3373	0.53724	0.0090164
Threonine*	AYFVYJQAPQTCCC-GBXIJSLDSA-N	1.4467	1.2792	1.8691	1.242	1.2061	0.0096721
L-alanine*	MUBZPKHOEPUJKR-UHFFFAOYSA-N	1.0679	1.0122	0.8546	1.0102	0.96143	0.011803
Uracil*	ISAKRJDGNUQOIC-UHFFFAOYSA-N	0.17897	0.366	0.22317	0.3448	0.70605	0.015902
Cholic acid*	BHQCQFFYRZLCQQ-OELDTZBJSA-N	1.5243	1.3972	1.8978	1.3157	1.2664	0.020328
Glycerol*	PEDCQBHIVMGVHV-UHFFFAOYSA-N	1.1775	1.1305	1.5212	1.0639	1.0054	0.021148
Myristic acid*	TUNFSRHWOTWDNC-UHFFFAOYSA-N	0.05460	0.46469	0.07072	0.5799	0.56785	0.023279
Cholesterol*	HVYWMOMLDIMFJA-DPAQBDIFSA-N	0.52261	0.77314	0.17624	0.7276	0.70976	0.027377
Tagatose*	LKDRXBCSQODPBY-OEXCPVAWSA-N	1.1157	1.245	1.38	1.1698	1.113	0.028852
Phenylethylamine*	BHHGXPLMPWCGHP-UHFFFAOYSA-N	1.0647	1.5462	1.0064	1.4544	1.5756	0.032787
Hypoxanthine*	FDGQSTZJBFJUBT-UHFFFAOYSA-N	0.09652	0.28394	0.11887	0.2665	0.58207	0.032951
Palmitic acid*	IPCSVZSSVZVIGE-UHFFFAOYSA-N	0.49285	0.74893	0.58984	0.7380	0.70058	0.037049
L-norleucine*	LRQKBLKVPFOOQJ-YFKPBYRVSA-N	0.8472	0.77466	0.93985	0.7905	0.76421	0.039508
L-valine*	KZSNJWFQEVHDMF-BYPYZUCNSA-N	0.77076	0.70997	0.99092	0.6778	0.72327	0.042951
Sorbose*	LKDRXBCSQODPBY-AMVSKUEXSA-N	0.6984	1.0726	0.90204	1.0223	0.96654	0.044754
Succinic acid*	KDYFGRWQOYBRFD-UHFFFAOYSA-N	2.0091	1.9716	2.2865	1.8752	1.7868	0.044918
Linoleic acid*	OYHQOLUKZRVURQ-HZJYTTRNSA-N	0.98508	0.91011	0.82359	0.9529	0.90628	0.04623
Iminodiacetic acid*	NBZBKCUXIYYUSX-UHFFFAOYSA-N	0.34183	0.51782	0.39691	0.5116	0.49391	0.053115
Glycine*	DHMQDGOQFOQNFH-UHFFFAOYSA-N	0.83115	0.76955	0.25675	0.7352	0.7	0.063115
Arabitol*	HEBKCHPVOIAQTA-QWWZWVQMSA-N	1.2402	1.3004	1.1307	1.2202	1.1702	0.071967
Threitol*	UNXHWFMMPAWVPI-QWWZWVQMSA-N	1.2402	1.3004	1.1307	1.2202	1.1702	0.071967
Name*	-	1.4617	1.2793	1.4356	1.261	1.2628	0.084426

Cellobiose2*	DLRVVLDZNNYCBX-ABXHMFFYSA-N	1.0521	1.1194	0.36794	1.177	1.2056	0.087705
Cellobiose1*	DLRVVLDZNNYCBX-ABXHMFFYSA-N	1.0965	1.1616	0.27576	1.2031	1.2329	0.08918
L-lactic acid*	JVTAAEKCZFNVCJ-REOHCLBHSA-N	0.38064	0.34109	0.42171	0.3403	0.54515	0.098361
Nicotinic acid*	PVNIIMVLHYAWGP-UHFFFAOYSA-N	0.2932	0.42574	0.04331	0.4496	0.80456	0.10361
Glucose*	WQZGKKKJIJFFOK-GASJEMHNSA-N	1.1379	1.0103	1.4604	0.9582	1.0583	0.10869
Talose*	WQZGKKKJIJFFOK-WHZQZERISA-N	0.98914	0.89045	1.2805	0.8523	0.97672	0.11852
Phosphoric acid*	NBIIXXVUZAFLBC-UHFFFAOYSA-N	0.94288	0.89642	1.0701	0.8994	0.85042	0.13213
Oleic acid*	ZQPPMHVWECSIRJ-KTKRTIGZSA-N	0.82774	0.8867	0.87563	0.8825	0.84222	0.13557
Allose*	WQZGKKKJIJFFOK-IVMDWMLBSA-N	0.73394	0.97555	0.93891	0.9914	0.93876	0.13656
Altrose*	WQZGKKKJIJFFOK-VSOAQEOCSA-N	0.50595	0.92205	0.39772	1.089	1.0305	0.13852
Benzoic acid*	WPYMKLBDIGXBTP-UHFFFAOYSA-N	1.0249	0.91542	0.77027	0.9312	0.89526	0.14951
Lactose*	GUBGYTABKSRVRQ-DCSYEGIMSA-N	0.87631	0.91851	0.98755	1.1343	1.1067	0.15033
Melibiose*	DLRVVLDZNNYCBX-ABXHMFFYSA-N	0.88773	0.92323	1.0214	1.1454	1.1147	0.15918
Glycolic acid	AEMRFAOFKBGASW-UHFFFAOYSA-N	1.7136	1.7406	1.9566	1.6458	1.5606	
DL-isoleucine	AGPKZVBTJJNPAG-UHFFFAOYSA-N	0.26715	0.26613	0.33241	0.4377	0.41604	
Glyceric acid	RBNPOMFGQQGHHO-UWTATZPHSA-N	0.23877	0.2287	0.17023	0.2314	0.52183	
L-serine	MTCFGRXMJLQNBG-REOHCLBHSA-N	0.90573	0.80824	1.1704	0.7656	0.72921	
Thymine	RWQNBRDOKXIBIV-UHFFFAOYSA-N	0.2904	0.60831	0.13184	0.6657	0.85964	
Malonic acid	OFOBLEOULBTSOW-UHFFFAOYSA-N	0.91188	0.81386	0.88259	0.7758	0.74087	
Methionine	FFEARJCKVFRZRR-BYPYZUCNSA-N	1.0606	1.0069	1.0096	0.9487	0.90453	
Aspartic acid	CKLJMWTZIZZHCS-REOHCLBHSA-N	0.47085	0.41676	0.57776	0.7306	0.74922	
4-guanidinobutyric acid	TUHVEAJXIMEOSA-UHFFFAOYSA-N	0.23097	0.3392	0.13804	0.3232	0.32754	
Alpha ketoglutaric acid	KPGXRSRHYNQIFN-UHFFFAOYSA-N	0.70868	0.79797	0.85838	1.0383	0.98219	
Glutamic acid	WHUUTDBJXJRKMK-VKHMYHEASA-N	0.82114	0.7985	1.0603	0.9751	1.1655	
5-aminovaleric acid	JJMDCOVWQOJGCB-UHFFFAOYSA-N	0.41256	0.68247	0.15453	0.7494	1.0804	
Lyxose	SRBFZHDQGSBBOR-AGQMPKSLSA-N	0.07967	0.07130	0.08088	0.3750	0.61504	
6-deoxy-D-glucose	SHZGCJCMOBCMKK-GASJEMHNSA-N	0.77237	0.79383	0.65727	0.7932	0.7504	
xylitol	HEBKCHPVOIAQTA-NGQZWQHPSA-N	0.5054	0.63101	0.41881	0.6327	0.65337	
Galactose	WQZGKKKJIJFFOK-SVZMEOIVSA-N	0.66763	0.96068	0.84803	0.9845	0.9307	

Tyramine	DZGWFCGJZKJUFP-UHFFFAOYSA-N	0.69223	0.82033	0.59041	0.8402	0.79394	
Lysine	KDXKERNSBIXSRK-YFKPBYRVSA-N	0.42108	0.69937	0.49143	0.6578	0.6221	
Tyrosine	OUYCCCASQSFEME-QMMMGPOBSA-N	0.79626	0.71207	0.74887	0.6674	0.63055	
Allo-inositol	CDAISMWEOUEBRE-UHFFFAOYSA-N	1.194	1.1	1.3715	1.0443	1.0161	
Sucrose	CZMRCDWAGMRECN-UGDNZRGBSA-N	0.58274	0.52774	0.69347	1.0782	1.026	

(International Chemical Identifiers (InChI) and standard InChI hashes (InChIKey); FC = Fold change)

Chapter 5

Prebiotic green banana resistant starch and probiotic *Bacillus coagulans* spores synbiotic supplementation ameliorates gut inflammation in mouse model of IBD

5.1 Abstract

The research goal is to develop dietary strategies to help address the growing incidence of IBD. This study has investigated the effectiveness of green banana resistant starch (GBRS) and probiotic *B. coagulans* MTCC5856 spores for amelioration of dextran-sulfate sodium (DSS)-induced colitis in mice. Eight-week-old C57BL/6 mice were fed normal chow diet supplemented with either *B. coagulans*, GBRS or synbiotic combination. After 7-days supplementation, colitis was induced by adding 2% DSS in drinking water for 7 days while continuing the supplemented diets. Animal health was monitored and after the 14 days all animals were sacrificed to measure the biochemical and histochemical changes associated with each supplement type. Synbiotic supplementation alleviated the disease activity index (DAI) and histological damage score (-67%, 8.8 respectively) more adequately than *B. coagulans* (-52%, 10.8 respectively) or GBRS (-57%, 13.6 respectively) alone. Compared to DSS-control Synbiotic supplementation significantly ($P<0.0001$) maintained expressions of tight junction proteins. Moreover, synbiotic effects accounted for ~ 40% suppression of IL-1β and ~29% increase in IL-10 levels in serum while, also reducing C-reactive protein (-37%) to that of DSS-control. While, *B. coagulans* alone could not induce additional levels of short-chain fatty acid (SCFA) production beyond the caecum, the synbiotic combination with GBRS resulted in substantial increased SCFA levels across the whole length of the colon. The amelioration of overall inflammatory parameters in this experimental IBD model by synbiotic supplementation with *B. coagulans* and GBRS supports researching its application in mitigating inflammation in human IBD.

5.2 Introduction

Although the pathogenesis of IBD that encompasses CD and UC, still remains unclear, emerging evidence substantiates the role of interaction of genetic, environmental and immunological factors. Perturbations in the composition of the gut microflora (dysbiosis) are associated with the pathogenesis of IBD (88, 91, 544). As diet is a major factor influencing the enteric microflora, numerous research projects have considered the role of specific nutrients in the development of IBD. The western diet, characterised by low intake of dietary fibre, has been linked with increased risk of IBD in several studies (9) and implicated in leading to gut dysbiosis that further aggravates gut inflammation. In this regard, prebiotic dietary fibre and probiotics are considered as critical components of dietary improvements in the context of IBD as both bioactive agents function to suppress inflammation via a number of proposed mechanisms (26, 242, 545). Hence, various probiotic and prebiotic agents are being increasingly explored to treat IBD in humans (242, 546, 547).

A number of factors influence the beneficial effects of probiotics. Their survival in delivery formats, including functional foods, is required as well as during gastric transit in order to exert health effects of the live organism (548). In this context, *Bacillus* species are a growing research focus due to the ability of their heat-stable spores to survive gastric transit, harsh manufacturing and storage temperatures and delivery formats that potentially involve hot foods (355, 425). Furthermore, *Bacillus* strains are known to exert therapeutic effects owing to their ability to induce immune responses and to produce antimicrobial peptides that help mitigate inflammation (425). *Bacillus coagulans* MTCC 5856 spores, specifically, have been shown to survive during harsh processing and storage conditions of functional foods (43), survive gastric transit, induce excellent immunomodulatory effects *in-vitro* (42) and exhibit beneficial effects in therapeutic management of clinical diarrhea (48).

An additional factor that may influence the beneficial effects of probiotics are their ability to generate fermentation products that influence the composition of the gut microbiome and thus potentially the health of the host. Bacteriocin and organic acid production are two possible characterized antimicrobial products of probiotics that can influence and stabilize the gut microbiome (549, 550). Other intermediate metabolites, including short-chain fatty acids (SCFAs) produced as a consequence of bacterial fermentation of prebiotic in the gut, have been affirmed to exert beneficial effects on the host (545, 551). The probiotic effect could therefore be potentiated by co-supplementation with

prebiotic dietary fibre that can be metabolised and fermented by the administered probiotic, as well as by beneficial microflora in the gut, to direct the shift of immune markers from pro-inflammatory to anti-inflammatory phenotype (552). This combination of probiotic and prebiotic factors can be referred to as synbiotic and potentially offers greater success of colonisation and survivability of the beneficial bacteria owing to the synergy than either probiotic or prebiotic alone (442). *B. coagulans* MTCC5856 are known to ferment variety of plant fibres. This therefore makes them a candidate for probiotics in synbiotic combination with plant prebiotic fibres.

Resistant starch (RS) is now classified as a type of dietary fibre (553). RS is defined as the sum of starch and the degradation products of starch that, on average, reaches the large intestine of healthy adult humans. Numerous clinical studies have successfully demonstrated the beneficial effects of RS in colonic health (553, 554). RS, derived as a ground flour from dried green banana (GB), has been demonstrated to prevent intestinal inflammation (76) and modulate oxidative stress (77) in animal models of colitis and impart anti-diarrhoeal effects in children (78, 79). Due to high RS content and nutrition value, GB flour is being increasingly incorporated in food products to increase functional properties such as stabilising emulsions (555-557). Synbiotic functional foods, targeted towards improving gut health by carrying both probiotic and prebiotic, could be of high interest for health food applications such as for mitigating inflammation in IBD patients. However improved knowledge on possible health effects, dosage response and mechanisms is needed to support such developments.

For this research it was hypothesised that supplementing the diet with probiotic *B. coagulans* MTCC5856 spores, prebiotic green banana resistant starch (GBRS) flour and its synbiotic combination may ameliorate the severity of DSS-induced colitis in mice model of IBD. This is the first report to investigate the anti-inflammatory potential of GBRS flour prepared from green lady finger bananas. This flour has all the fibre from green banana fruit. The fibre levels are very high being greater than 50% w/w owing to high RS content in addition to other nutritional components such as 5-hydroxytryptophan (5-HTP) (72). This study aimed to investigate the efficacy of pre-conditioning the gut with diet supplemented with *B. coagulans* spores, GBRS, both individually and in synbiotic combination prior to colitis-induction in ameliorate the severity of colitis in mice and analyse its underlying mechanism.

5.3 Materials and methods

5.3.1 Probiotic Bacteria and Prebiotic Dietary Fibre

LactoSpore® containing probiotic strain *Bacillus coagulans* MTCC 5856 (6×10^9 spores/gm) was produced by Sami Labs Limited (Bangalore, India) and supplied by Sabinsa Corporation (Australia). Prebiotic green banana resistant starch (GBRS) was supplied by Natural Evolution™, Australia (Appendix II).

5.3.2 Animals

Fifty C57BL/6J (seven week old) mice of both sexes of average weight 19g were obtained from the University of Tasmania animal breeding facility and housed in a temperature-controlled environment with a 12-h day/night light cycle. Individual body weights were assessed daily including over an initial acclimation period of seven days. All mice had *ad libitum* access to radiation-sterilised rodent feed pellets (Barastoc Rat and Mouse, Ridley AgProducts, Australia, Appendix III) and autoclaved tap water for drinking during experiments. All animal experiments were approved by the Animal Ethics Committee of the University of Tasmania [ethics approval number: A0015840 (Appendix IV)] and conducted in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (8th Edition, 2013). All efforts were made to minimize animals' suffering and to reduce the number of animals used.

5.3.3 Study Design and Treatments

Following 1 week of acclimation, mice at 8 weeks of age were randomly allocated into following 5 groups (n = 10 per group): (1) Healthy Control (HC), (2) DSS-control, (3) Probiotic *B. coagulans* MTCC 5856 (*B. coagulans*), (4) Prebiotic green banana resistant starch (GBRS) and (5) Synbiotic. The experimental design of the mice feeding trial is illustrated in Figure 5.1. Mice in HC and DSS-control groups received 4g chow mash (standard chow pellet blended with water). The *B. coagulans* group received 4 g chow mash supplemented with probiotic *B. coagulans* MTCC 5856 spores (2×10^9 CFU/day/mouse). The GBRS group received 4g chow mash supplemented with GBRS (400 mg/day/mouse). The Synbiotic group received 4 g chow each supplemented with *B. coagulans* MTCC 5856 spores (2×10^9 CFU/ day/mouse) and GBRS (400 mg/day/mouse). The chow mash was prepared fresh each day. The mice were single-caged throughout the experiment to measure the defined daily intake of respective treatments from prepared chow mash (4g). The mice were fed these

treatments for 14 days. Colitis was induced during the last 7 days of the experimental period by administering 2% dextran sulfate sodium (DSS; MP Biomedicals, colitis grade average molecular weight: 36,000-50,000) in drinking water of all groups except for non-colitic control mice which received normal drinking. Mice were sacrificed on day 15 by CO_2 asphyxiation.

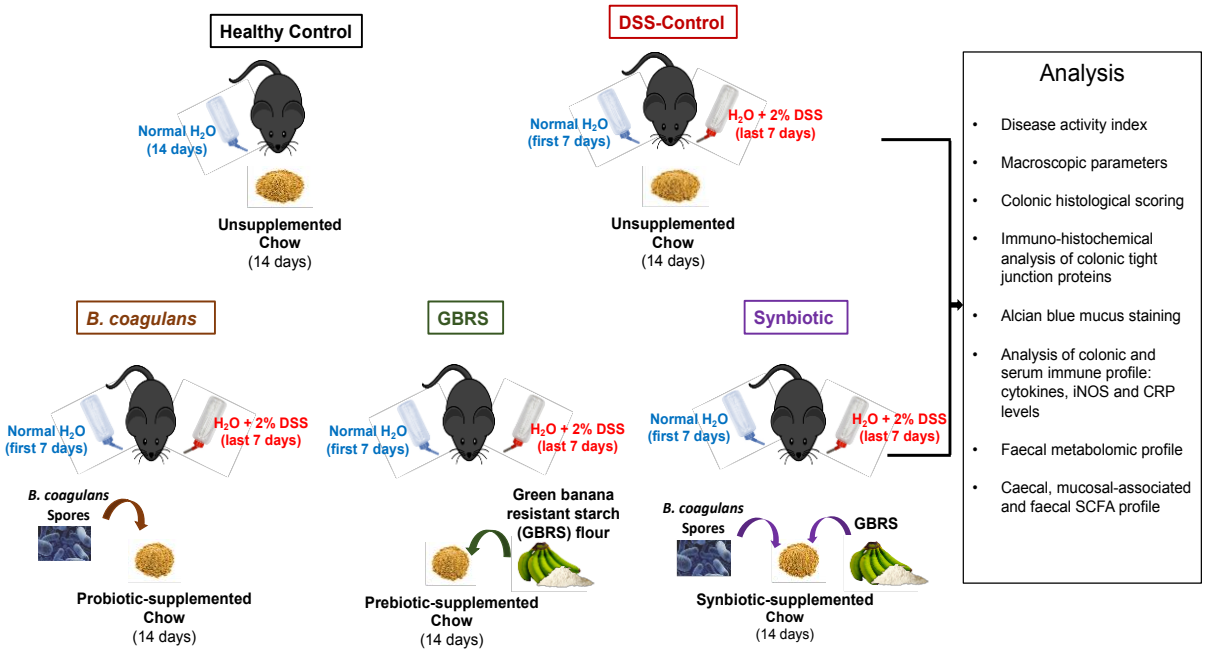


Figure 5.1. Experimental design of *in-vivo* feeding trial to analyse prophylactic efficacy of *B. coagulans* spores, GBRS and Synbiotic in DSS-induced acute colitis mice model. C57BL/6J mice (n = 10 per group) were fed chow supplemented with either *B. coagulans* spores, GBRS or their Synbiotic combination for 14 days. Colitis was induced by administration of 2% DSS in drinking water for last seven days.

5.3.4 Clinical Scoring and Histological Analysis

A Disease Activity Index (DAI) was determined daily in all mice by scoring for body weight, hemoccult reactivity or presence of gross blood and stool consistency during the week of DSS induction. DAI was determined by combining the scores from these three categories as detailed in Section 4.3.4 of Chapter 4 (552). Faecal samples were collected on day 14 and stored at −80 °C for metabolite analysis. After sacrificing the mice, the colons were dissected from the caecum to the anus as described previously. The mucosal and caecal contents were collected for metabolite profiling and stored at −80 °C. The collection, preparation and storage of colonic tissues for molecular analyses and histological staining as detailed previously in Chapter 4. For histological analysis, proximal and distal colon tissue sections (n = 8 per

group) were stained with H&E stain and graded blindly for the severity of tissue damage at distal and proximal regions as described previously (552).

5.3.5 Alcian Blue Staining

DSS-induced alterations in goblet cells, and subsequent depletion in synthesis and secretion of mucin glycoprotein (MUC2), were analysed by Alcian blue staining (ab150662 Alcian Blue, pH 2.5 (Mucin Stain), Abcam, Australia) following the manufacturer's instructions as previously described in Chapter 4 (4.3.5). The staining intensity (IOD) was assessed using Image Pro Plus 7.0 (Media Cybernetics, Inc., Rockville, MD, USA) and used for comparison among groups (506).

5.3.6 Immunohistochemical Detection of Tight Junction Proteins

Immunohistochemical detection of epithelial tight junction (TJ) proteins : ZO-1, occludin and claudin-1 was performed using a Rabbit specific HRP/DAB (ABC) Detection IHC kit (ab64261, Abcam, Australia) following the manufacturer's instruction and as previously described (552). Antibodies anti-ZO-1 (NBP1-85046, Novus Biologicals, Australia, 1:400); anti-occludin (NBP1-87402, Novus, 1:600) and anti-claudin-1 (NBP1-77036, Novus, 1 μg/mL) were used for incubating the colonic sections overnight at 4 $^{\circ}$C. Computer-assisted image analysis was performed with a Leica DM500 microscope (Leica Microsystems, Wetzlar, Germany), Leica ICC50 W camera (Leica Microsystems, Wetzlar, Germany), and Image Pro Plus 7.0 (Media Cybernetics, Inc., Rockville, MD, USA) software. The expression of tight junction (TJ) proteins: ZO-1, occludin and claudin-1 was blindly assessed by choosing random five fields on each slide (n = 4/group). Barrier TJ protein expressions and staining intensity in colonic epithelium was expressed as the percentage expression of a respective TJ protein.

5.3.7 Myeloperoxidase Activity

The extent of the inflammatory cell invasion in the colon was examined by the assessment of myeloperoxidase (MPO) activity (499). Weighed and snap frozen PC and DC specimens (n = 3) were analysed for MPO activity using a Myeloperoxidase Activity Assay kit (ab105136, colorimetric, Abcam®, Cambridge, UK) as described previously (552). The values are expressed as MPO activity units/g tissue.

5.3.8 Tissue Explant Culture and Cytokine Measurements

PC and DC colon tissues of mice from each group were cut, weighed and washed with cold PBS before transferring to a 12 well plate containing 0.5 mL/well of RPMI1640 culture medium (In Vitro Technologies Pty Ltd, Melbourne, Australia) supplemented with 10% *v/v* foetal calf serum (Gibco, Life Technologies Pty Ltd, Melbourne, Australia), penicillin (100 mU/L), and streptomycin (100 mg/L) (Sigma-Aldrich Pty Ltd, Sydney, Australia) as described previously (501). After 24 h of incubation, supernatant was collected from each well, centrifuged and stored at −80 °C until further analysis. Serum was collected from blood drawn by cardiac puncture at the end of the study for cytokine analysis.

The cytokine levels in colon tissue (n = 3) and serum (n = 3) were determined by immunoassay using a Bio-Plex Pro Mouse cytokine 23-plex kit (Bio-Rad #M60009RDPD, Bio-Rad Laboratories, Gladesville, NSW, Australia) following the manufacturer's instructions and concentrations analysed using a Bio-Plex 200 instrument (Bio-Rad) and Bioplex Manager software, version 6 (Bio-Rad Laboratories) respectively. For tissues, the cytokine levels were normalized by dividing the cytokine results (pg/mL) by the measured biopsy weight (g). The most significantly altered cytokines are presented as pg/g of tissue.

5.3.9 iNOS Activity

The expression of inducible isoforms of nitric oxide synthase (iNOS) in colonic epithelial cells in response to pro-inflammatory stimuli (508) was determined in PC and DC specimens using a Nitric Oxide Synthase Activity Assay kit (ab211084, Fluorometric, Abcam®, Cambridge, UK), following the previously described method (552). The results are expressed as iNOS activity mU/mg.

5.3.10 Serum C-Reactive Protein Analysis

The levels of C-reactive protein (CRP) in serum from respective groups (n = 3 samples/group) were analysed using Mouse C-Reactive Protein/CRP Quantikine Elisa kit (MCRP00, R and D Systems, Australia) following the manufacturer's instructions. The results are expressed as μg/mL.

5.3.11 Volatile SCFA Analysis

GC-MS analysis of 100–150 mg fresh weight (stored at −80 °C) of caecal, mucosal-associated and faecal samples (n = 5 per group) each was conducted for volatile SCFA profiling following the method described previously (510, 552). The GC-MS analysis was performed by Dr. David J. Beale (CSIRO), Dr. Avinash V. Karpe (CSIRO) and Dr. Shakuntala V. Gondalia (Swinburne University of Technology). Data analysis and interpretation was performed by the PhD candidate.

5.3.12 Metabolic Phenotyping Analysis

Untargeted metabolomic profiling of faecal samples (n = 5 per group) were performed using GC-MS analysis by Dr. David J. Beale (CSIRO), Dr. Avinash V. Karpe (CSIRO) and Dr. Shakuntala V. Gondalia (Swinburne University of Technology) as described previously (510, 552). Data analysis and interpretation was performed by the PhD candidate.

5.3.13 Statistical Analysis

The samples in the study were randomly chosen for all the analyses to avoid bias. All data are presented as means ± standard error of the mean (SEM). The statistical analysis was performed with the use of GraphPad Prism Software (Version 7.0, San Diego, CA, USA). The data were evaluated using One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test to determine statistical differences between the groups against the DSS-control samples. For the analysis of DAI and body weight changes during the experimental period, two-way ANOVA followed by Tukey's post-hoc test was used, setting treatment and the time as the variables. A p-value of < 0.05 was considered significant. A MetaboAnalyst (Version 4.0) data annotation approach and Kyoto Encyclopaedia of Genes and Genomes (KEGG) Pathway Database were used for the hierarchical clustering analysis and significance analysis for microarrays (SAM), along with the variable importance of projection (VIP) (517). The SAM and VIP methods are well-established statistical methods for metabolites and were used to select the most discriminant and interesting biomarkers (518).

5.4 Results

5.4.1 Effects of B. coagulans, GBRS and Synbiotic supplementation on clinical manifestations and macroscopic inflammatory markers

DSS-induction resulted in a progressive increase in colonic inflammation demonstrated by severe body weight loss and high DAI (Figure 5.2A, 5.2B). However, supplementation of *B. coagulans*, GBRS and Synbiotic treatments, started 7 days prior DSS-induction, attenuated the impact of the DSS damage and boosted the recovery of the treated animals. This is evidenced by the significant reduction in body weight loss and by lower incidences of diarrheic/bloody faeces, resulting in lower DAI values throughout the experiment in the treated groups when compared to untreated DSS-control group. At the end of the experiment on day 8, DAI was significantly ($P < 0.0001$) higher for DSS-control group (6.1 ± 0.5) compared to *B. coagulans* (2.9 ± 0.4, 52% reduction), GBRS (2.6 ± 0.4, 57% reduction) and Synbiotic (2.0 ± 0.2, 67% reduction) mice (Figure 5.2B). Moreover, probiotic, prebiotic and synbiotic supplementation significantly ($P < 0.0001$) reduced the loss of body weight compared to that of DSS-control group starting from day 6.

The macroscopic evaluation of colonic segments affirmed the remedial effects of all three treatments used in our study, as indicated by a substantial reduction in colon weight/body weight ratio (*B. coagulans*, 7.70±0.2; GBRS, 9.32±0.3 and Synbiotic, 8.32±0.3 mg/g) compared with DSS-control group (11.16±0.2 mg/gm) (Figure 5.2C). Relative spleen weights of DSS-control mice were markedly higher (0.08±0.004 g) than that of GBRS (0.063±0.002 g) and Synbiotic (0.062±0.003 g) mice (Figure 5.2D). *B. coagulans* had no effect on spleen weight reduction (0.068±0.004 g). *B. coagulans* (7.80±0.3 cm), GBRS (7.91±0.2 cm) and Synbiotic (8.09±0.2 cm) supplementation effectively prevented the colon shortening compared with the DSS-control group (6.80±0.3 cm) (Figure 5.2E, 5.2F). Additionally spleen enlargement, increased colon weight/body weight ratio and colon shortening was directly associated with intestinal inflammation and disease severity in experimental colitis models (499).

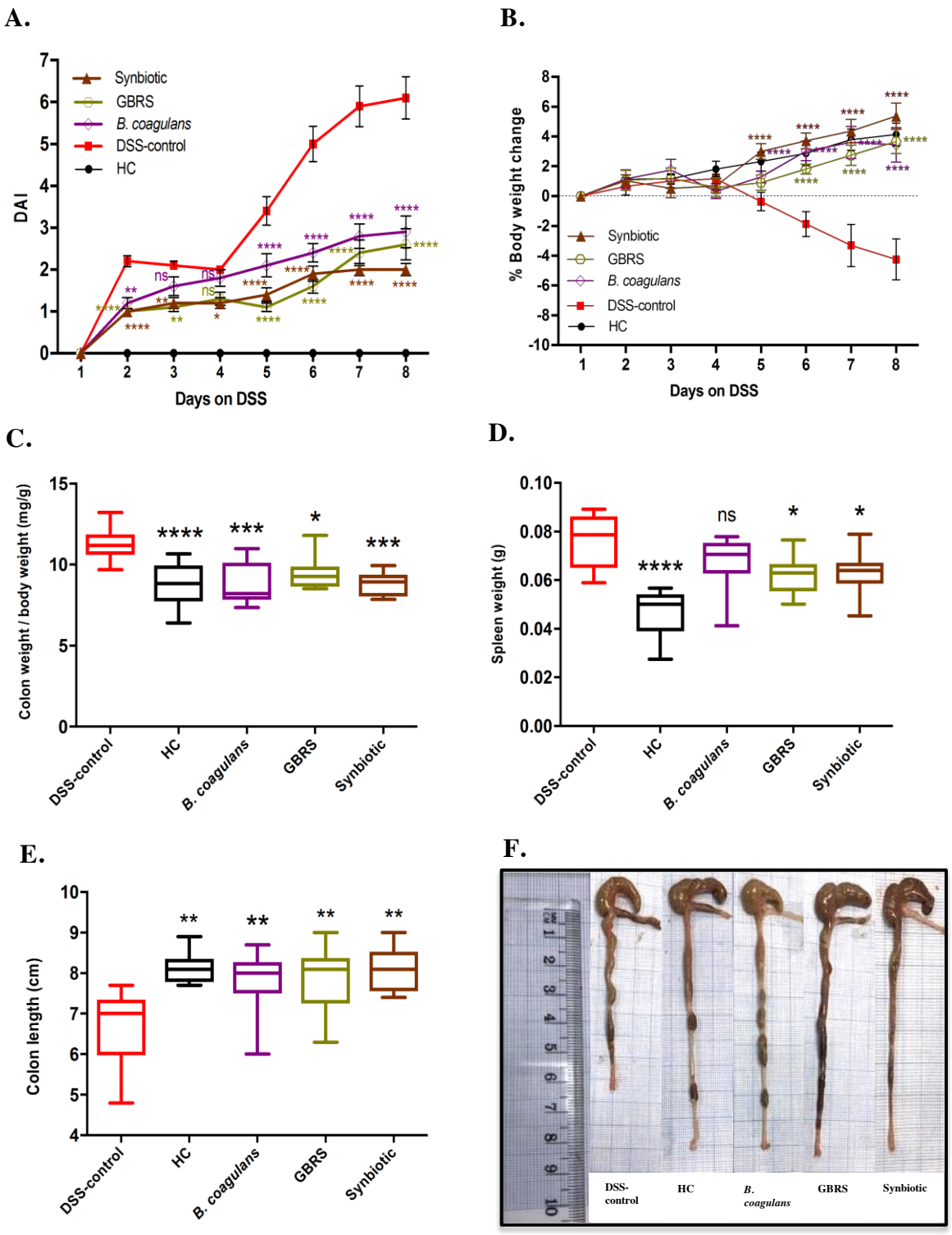


Figure 5.2. Effect of *B. coagulans* spores, GBRS and Synbiotic in DSS-induced colitis model. (**A**) Disease Activity Index (DAI), (**B**) % body weight change. Statistical significance among groups evaluated by two-way repeated-measures analysis of variance (ANOVA) followed by Tukey's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. DSS-control group and data expressed as mean ± SEM (n =10 per group). Colon weight/body weight ratio (**C**), Spleen weight (**D**), Colon length (**E**) and macroscopic appearance of colon (**F**). Data expressed as mean ± SEM (n =10 per group), evaluated by one-way ANOVA followed by Tukey's Test. NS = non-significant.

5.4.2 Effects of B. coagulans, GBRS and Synbiotic supplementation on histological alterations in colon

Histological (H&E staining) examination of proximal colon (PC) and distal colon (DC) sections of DSS-induced mice displayed histological damage with erosion or destruction of epithelium, crypt distortion, depletion of goblet cells, submucosal oedema and inflammatory cellular infiltration in the colon, mostly affecting distal section (Figure 5.3A). While, HC showed no signs of histological colon damage (score 0), DSS resulted in a cumulative damage score of 9.38±0.8 for PC and 17.1±0.4 for DC (Figure 5.3B). Supplementation with Synbiotic and *B. coagulans* induced protection against the damage, as evidenced by substantial retention of colonic structure, protection of crypts and goblet cells, and reduced infiltration of inflammatory cells which resulted in a significant overall reduction of cumulative histological scores of DC (8.8±0.5, 10.8±1.0 respectively). GBRS provided a partial but significant protection with a histological score of 13.6±0.7. In contrast, histology scores for PC demonstrated no statistically significant protection by the three treatments. MPO assay however, showed a substantial reduction in neutrophil infiltration in PC by synbiotic and *B. coagulans* compared with that of the DSS-colitic group. In DC, all three supplementations were successful in reducing the inflammatory cell infiltrate as determined by decreased MPO activity (Figure 5.3C) with *B. coagulans* and Synbiotic being more effective than GBRS.

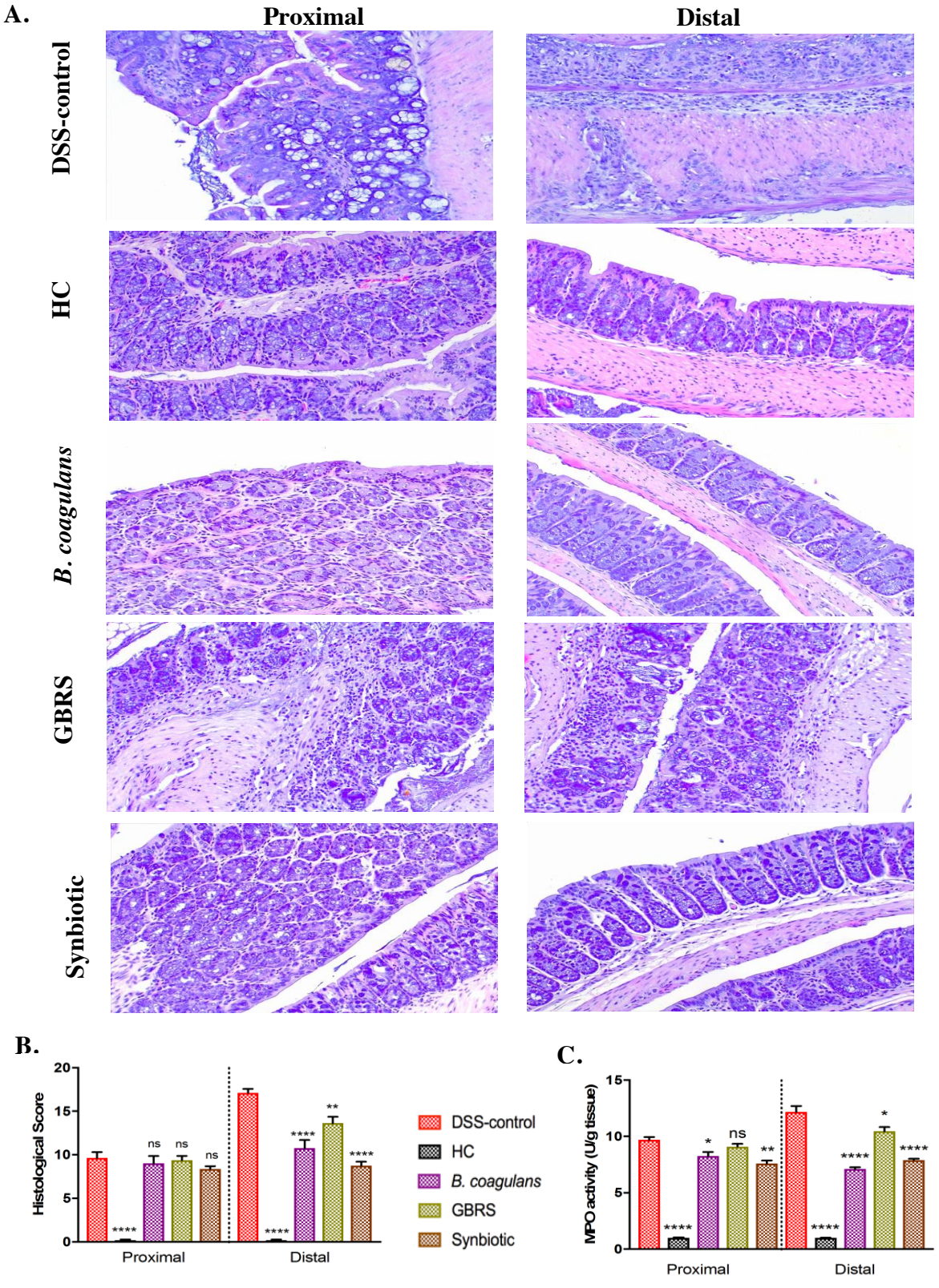


Figure 5.3. Effect of *B. coagulans* spores, GBRS and Synbiotic treatments on DSS-induced colon injury and inflammation. (**A**) Histological images of proximal and distal colonic tissues stained with hematoxylin and eosin at 20× for each experimental group. (**B**) Histological score calculated after microscopic analyses of proximal and distal sections of the colon. (**C**) Myeloperoxidase (MPO) activity in colonic tissues was determined by colorimetric assay. Results, expressed as mean ± SEM (n = 8 per group), were evaluated by one-way ANOVA followed by Tukey's test (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).

5.4.3 Effects of B. coagulans, GBRS and Synbiotic supplementation on goblet cells and colonic tight junction barrier

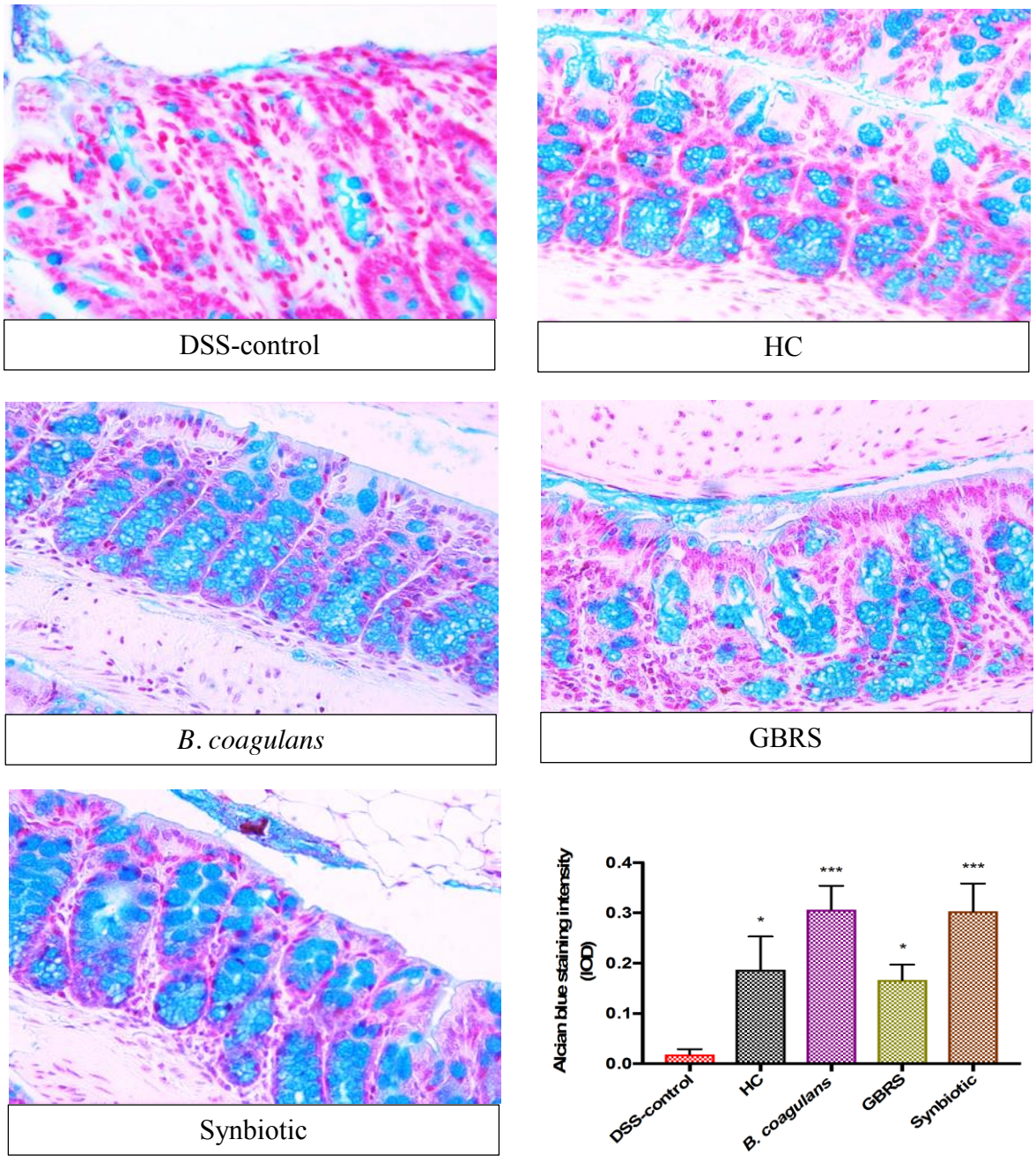


Figure 5.4. Effect of *B. coagulans* spores, GBRS and Synbiotic on goblet cells. The paraffin embedded sections were stained with Alcian Blue to detect changes in goblet cells and in production of mucus in distal colonic tissue in each experimental group (40×) and staining intensity (IOD) of respective group is illustrated in the graph. Results expressed as mean ± SEM (n = 4 per group), evaluated by one-way ANOVA followed by Tukey's test (*$P < 0.05$, ***$P < 0.001$).

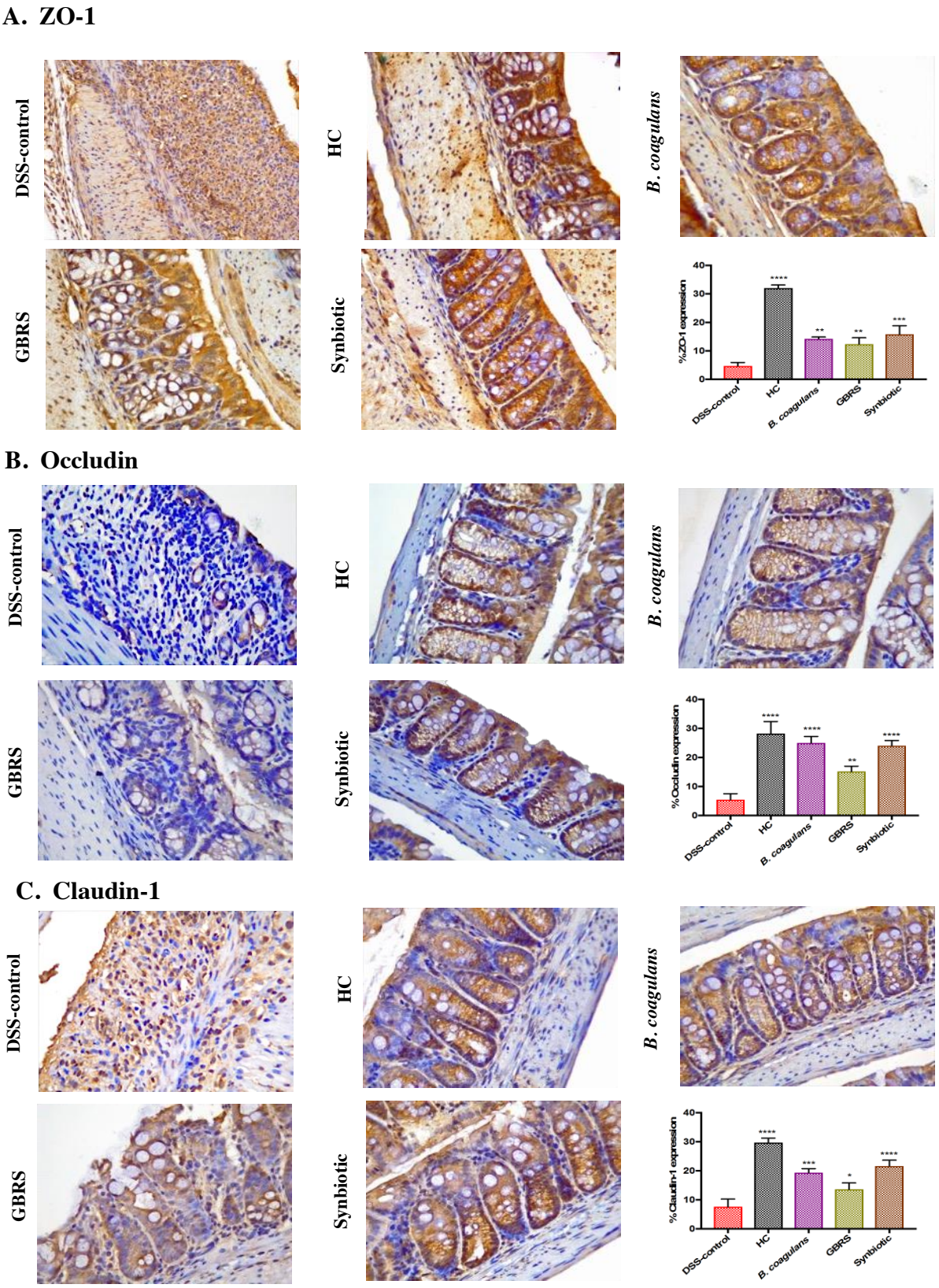


Figure 5.5. Effects of *B. coagulans* spores, GBRS and Synbiotic on expression of epithelial tight junction proteins. Immunohistochemical detection of (**A**) ZO-1, (**B**) Occludin and (**C**) Claudin-1 and its respective percentage of expression in colon at 40×. Data expressed as mean ± SEM (n = 4 per group) and statistical significance among groups evaluated by one-way ANOVA followed by Tukey's test *P < 0.05, **p < 0.01, ***P < 0.001, ****P < 0.0001 vs. DSS-control group.

Staining with Alcian blue was performed to examine the effect of supplementation on DSS-induced alterations in the mucus secretion by goblet cells. Significantly high mucus staining with Alcian blue was detected in colon sections of mice supplemented with *B. coagulans* and Synbiotic with moderate capacity of GBRS suggesting induction of high secretion levels of mucus in DSS-challenged mice that received supplementations (Figure 5.4). In comparison, in DSS-control colon sections, the goblet cells were almost entirely destroyed.

Immunohistochemical analysis was performed to investigate the impact of supplementation on assembly of the TJs and the integrity of the intestinal barrier. The presence of the TJ proteins- ZO-1, occludin and claudin-1 were analysed for on the colonic tissue sections (Figure 5.5). In HC sections, ZO-1 staining (Figure 5.5A) was more intense in the apical tight junction complex, both at the surface and in the crypts. Occludin (Figure 5.5B) and claudin-1 (Figure 5.5C) proteins stained more strongly at the basolateral membrane of the crypts, and also showed their presence at the crypt surface. In DSS-control sections however, such signals were weak or totally absent in line with previous reports (519, 520), indicating a low percentage of TJ protein expression. *B. coagulans* and Synbiotic supplementation, however, effectively maintained the basolateral and partial apical staining of ZO-1, occludin and claudin-1 in DSS-induced mice. GBRS only displayed partial maintenance of ZO-1 staining, although the effect was less noticeable for occludin and claudin-1. In contrast, Synbiotic supplementation significantly maintained the TJ patterns similar to that of HC sections, indicating a high level of protection of the integrity of the epithelium.

5.4.4 Immunomodulatory effects of B. coagulans, GBRS and Synbiotic supplementation on immune markers

B. coagulans, GBRS and Synbiotic supplementation improved the altered immune responses induced by DSS supporting their immunomodulatory and anti-inflammatory effects (Figure 5.6). In comparison with the DSS-control group, all three treatments substantially reduced the tested pro-inflammatory cytokine levels of IL-1α, IL-1β, IL-6, IL-12, TNF-α, IFN-γ in PC and DC segments. However, no significant effect of supplementations was noted on levels of the other cytokines (Supplementary Figure SF5.1). Supplementation with *B. coagulans* alone and Synbiotic proved effective in reducing the levels of all the cytokines tested in comparison with DSS-control mice. There was a pronounced reduction in increases

of IL-1β (-60%), IL-6 (-62%), TNF-α (-66%) and IFN-γ (-73%) levels by *B. coagulans* supplementation in comparison with that of GBRS (IL-1β: -36%, IL-6: -35%, TNF-α: -46%, IFN-γ: -57%). However, GBRS had no significant effect on the levels of IL-1α, IL-12 and TNF-α in DC. The Synbiotic supplementation proved effective in reducing the levels of all pro-inflammatory cytokines, in comparison with elevated cytokine levels in the DSS-control but, displayed greater reduction in the levels of IL-6 (-78%), IL-12 (-56%) and IFN-γ (-71%) compared with GBRS supplementation and greater suppression in the level of IL-1β (-75%) compared to *B. coagulans*.

Serum cytokines indicative of immunomodulatory effects also followed a similar trend (Figure 5.6G-I). Synbiotic significantly decreased pro-inflammatory serum cytokine levels of IL-1β (50.1±4.6 pg/mL) and IL-12 (146.4±7.4 pg/mL) while, concomitantly increasing anti-inflammatory IL-10 (181.7±6.1 pg/mL) levels compared with the DSS-control group (IL-1β: 82.8±4.1 pg/mL, IL-12: 246.6±6.0 pg/mL and IL-10: 140.5±6.5 pg/mL). While, *B. coagulans* and GBRS supplementations alone were not effective in reducing increased serum IL-1β (66.1±7.0 and 59.38±5.0 pg/mL respectively), significant reduction in IL-12 (179.3±12.3 and 169.5±11.1 pg/mL respectively) was achieved relative to that of the DSS-control. No significant effect was observed for other serum cytokines (Supplementary Figure SF5.1). DSS-induction elevated iNOS activity in both PC and DC in response to the pro-inflammatory stimulus, in line with the previous report (508). Synbiotic and *B. coagulans* lowered the iNOS activity significantly, while GBRS had no effect. Moreover, compared to high CRP levels in DSS-control (14.81±0.6 μg/mL), *B. coagulans* (10.31±0.3μg/mL), GBRS (10.61±0.7 μg/mL) and Synbiotic (9.4±0.2 μg/mL) reduced the serum CRP levels. Synbiotic and *B. coagulans* supplementations induced normalisation of CRP levels and were statistically similar to that of HC levels (8.3±0.5 μg/mL). These observations indicate that a combination of probiotic spore and prebiotic GBRS resulting a desirable level of immunomodulatory activity.

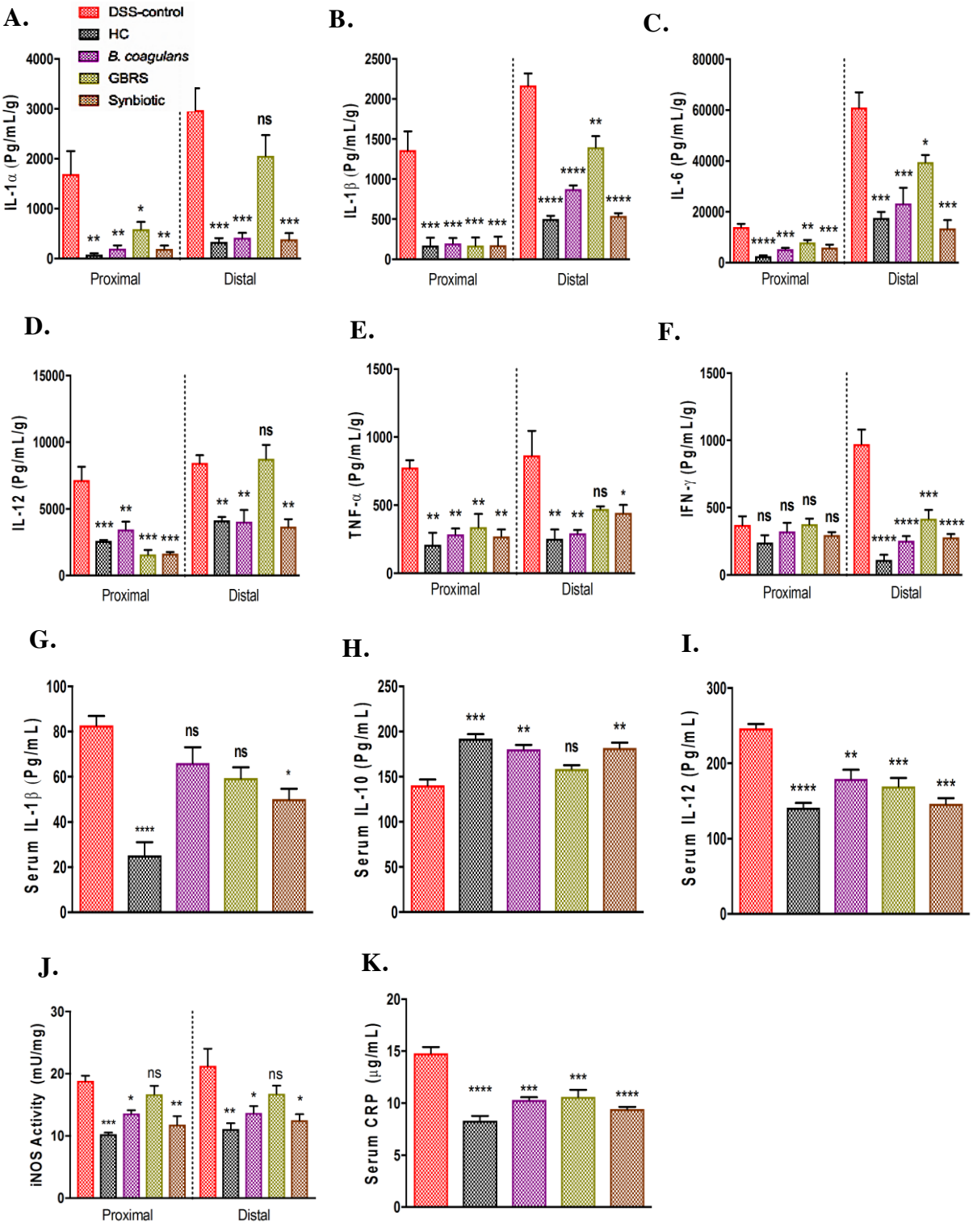


Figure 5.6. Effect of *B. coagulans* spores, GBRS and Synbiotic on immune markers in colon tissues and blood serum. Protein levels of cytokines including (**A**) IL-1α, (**B**) IL-1β, (**C**) IL-6, (**D**) IL-12, (**E**) TNF-α, (**F**) IFN-γ in proximal and distal colon explants as well as cytokine levels of (**G**) IL-1β, (**H**) IL-10, and (**I**) IL-12 in blood serum were analysed by Bio-plex. iNOS activity in colon tissues (**J**) measured by NOS activity assay and CRP levels in serum (**K**) by ELISA. Statistical significance among groups evaluated by one-way ANOVA followed by Tukey's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs. DSS-colitic group and data expressed as mean ± SEM ($n = 3$ per group).

5.4.5 Effects of B. coagulans, GBRS, and Synbiotic supplementation on alteration of faecal metabolic profile

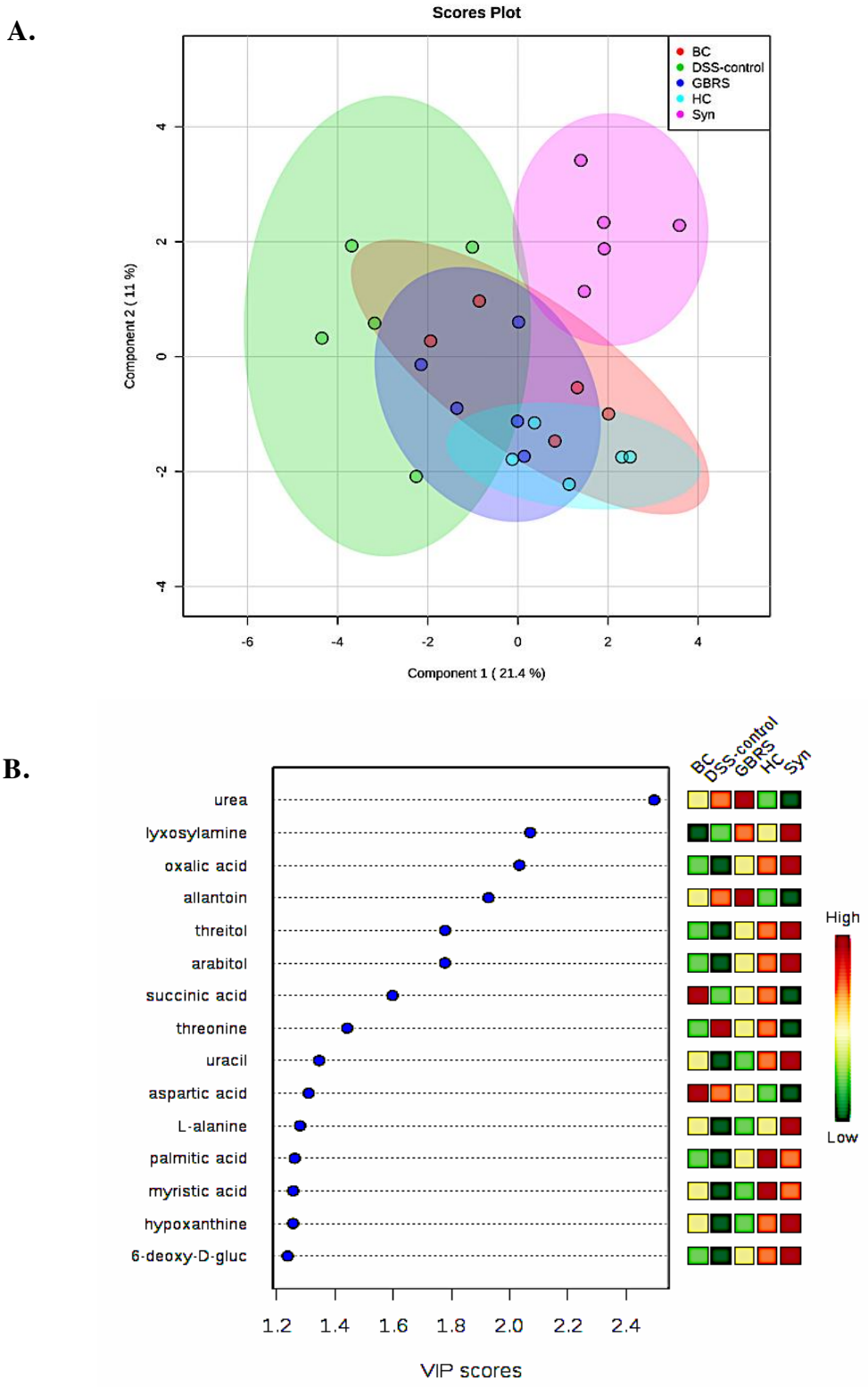


Figure 5.7 Effect of *B. coagulans* spores, GBRS and Synbiotic on metabolic modulations in DSS-induced colitic mice. (**A**) 2D-PLS-DA plot showing spatial division among groups that received different supplementations, DSS-control mice that received no supplementation and HC. (**B**) Key compounds separating the groups are ranked based on variable importance projection (VIP) score plot from PLS-DA analysis. (BC-*B. coagulans*, Syn-synbiotic).

Faecal samples were analysed by GC-MS platform to gain an untargeted overview of alterations in dominant gut metabolites induced by *B. coagulans*, GBRS and Synbiotic supplementations in DSS-treated mice. The analysis detected a total of 61 metabolites belonging to different functional groups such as sugars, amino acids, volatile fatty acids and biogenic amines. A supervised partial least squares-discriminant analysis (PLS-DA) was performed to evaluate metabolic phenotyping of each experimental group (Figure 5.7A). The remoteness between the samples from HC and DSS-control indicates a clear distinction in metabolic patterns between the groups. Among the supplemented groups, samples from *B. coagulans* and GBRS clusters overlapped with each other, and with that of HC, and partially with DSS-control. Synbiotic cluster showed clear divergence relative to that of DSS-control samples suggesting its potential to induce marked changes in the metabolic profile. Combination of PLS-DA (R2Y= 0.803 (P = 0.01), Q2 = 0.521), VIP scores (Figure 5.7B) and SAM enabled us to identify potential biomarkers. The results showed 61 metabolites with 28 statistically significant compounds contributing to the clustering, with their significance analysis for microarrays (SAM) scores fold changes and International Chemical Identifiers (InChI) and standard InChI hashes (InChIKey IDs) listed in Supplementary Table ST5.1. Key metabolic markers making a significant contribution were identified by VIP analysis as displayed in Figure 5.7B. Among these identified metabolites, substantial differences in the patterns between DSS-control and HC were particularly noted for allantoin, threitol, arabitol, uracil, aspartic acid, palmitic acid, myristic acid, hypoxanthine and 6-deoxy-D-glucose. Synbiotic supplementation generated reduction in the metabolic alterations induced by DSS (Figure 5.7B and Table ST5.1).

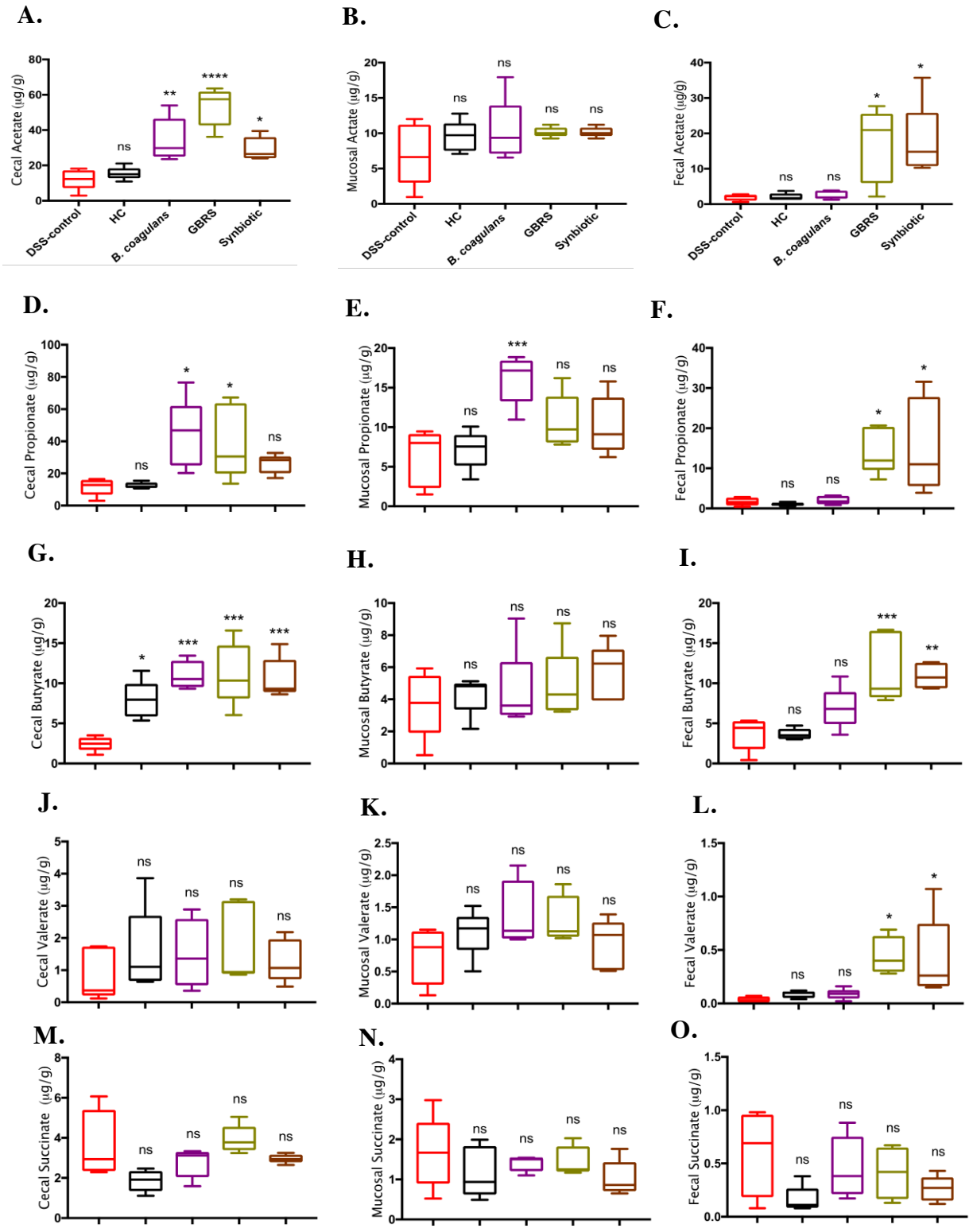


Figure 5.8. Effects of *B. coagulans* spores, GBRS and Synbiotic in modulating SCFA concentrations in caecal, mucosal-associated and faecal contents in DSS-induced colitis. Caecal- acetate (**A**), propionate (**D**), butyrate (**G**), valerate (**J**), succinate (**M**); mucosal-associated acetate (**B**), propionate (**E**), butyrate (**H**), valerate (**K**), succinate (**N**) and faecal- acetate (**C**), propionate (**F**), butyrate (**I**), valerate (**L**), succinate (**O**). Statistical significance among groups evaluated by one-way ANOVA followed by Tukey's test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 vs. DSS-colitic group and data expressed as mean ± SEM (*n* = 5 per group). ns = non-significant.

5.5 Discussion

Application of dietary strategies to prevent the onset or reduce severity of IBD is gaining momentum. The mechanisms that contribute to managing IBD appear to act through modulating cytokine responses, epithelial integrity and gut microbiota (521, 522). The results of the present study clearly indicated that pre-conditioning of gut with synbiotic supplementation carrying probiotic and prebiotic components, prior to DSS-induction, markedly reduced the symptoms and severity of DSS-induced colitis in the mouse model. The results supported the anti-inflammatory potentials of both the probiotic *B. coagulans* MTCC5856 and GBRS supplement ingredients. However, the effect was noted to be more profound with synbiotic supplementation as illustrated by its ability to prevent the clinical manifestations, macroscopic, histological, biochemical, metabolic and immune parameter changes in the DSS-induced mice. Synergistic action between the two bioactive components could account for such the enhanced beneficial effect.

During feeding the supplementation of DSS-induced mice with *B. coagulans*, GBRS and Synbiotic significantly ($P < 0.0001$) lowered the DAI scores observed by the marked reduction in body weight loss and lower incidences of diarrheic/ bleeding faeces compared to that of DSS-control mice (Figure 5.2). Green banana-supplemented diets (79) and *B. coagulans* spores (48) have each previously been shown to reduce clinical diarrheal episodes in line with the observations on DDS-induced mice in the current study. The anti-diarrheic effect of GBRS could be due to its high RS content that, upon reaching the caecum/colon, is metabolized by bacteria to SCFAs (77). These in turn, stimulate salt and water absorption, provide energy and induce a trophic effect on the colon (79). The ability of *B. coagulans* to elicit an anti-diarrheic effect could be via several proposed mechanism that include suppression and binding of pathogenic bacteria, improvement of the epithelial barrier function and alteration of the immune activity of the host (48). Synbiotic supplementation that combines these effects should stimulate more profound efficacy outcomes against manifestations of IBD, as supported by the observations in the current study.

The animal study allowed other observations not easily possible in clinical trials. A potential synbiotic outcome was demonstrated from improvement in histology of the colon (Figure 5.3A, 5.3B) compared to the DSS-control as well as to the *B. coagulans* and GBRS supplementations alone. Synbiotic supplementation also showed marked protection to the

colonic epithelial architecture by alleviating crypt disruption, loss of goblet cells, submucosal oedema and inflammatory infiltrates induced by DSS. In other biomarkers of IBD activity synbiotic supplementation caused significant reduction ($P < 0.0001$) in MPO activity (Figure 5.3C) especially in the DC section compared to that of DSS-control. Neutrophil-myeloperoxidase is an enzyme that catalyses production of reactive oxygen species and is increased in the mucosa of patients with IBD (558). The level of MPO activity is directly proportional to the neutrophil concentration and thus is an index of neutrophil infiltration and inflammation (520). MPO activity may cause oxidative damage to host tissue and induce or perpetuate inflammation. MPO is an important diagnostic and prognostic tool in assessing IBD status (558). This current research found that colonic MPO activity was markedly increased in DSS-control mice, and that synbiotic supplementation significantly reduced this effect in both PC and DC. This suggest that synbiotic supplementation has an anti-inflammatory effect that in analogous to the histological evidence of protection.

Synbiotic supplementation in this study, followed in efficacy by *B. coagulans* and GBRS alone, were also effective in protecting the TJ proteins (ZO-1, occludin and claudin-1) in DSS-induced mice (Figure 5.5). Disruption of intestinal epithelial TJs and impaired epithelial barrier function is a prominent event in the pathogenesis of clinical colitis that further promotes dysregulated immune reactions, thus aggravating gut inflammation. TJs maintain the epithelial barrier function by sealing the intracellular spaces between adjoining epithelial cells, thus restricting paracellular movement of harmful substances across intestinal mucosa (99). Our data shows that synbiotic supplementation exerted a marked protective effect on the barrier integrity by maintaining the expressions of the TJ proteins, thereby reducing the severity of gut inflammation. Moreover, Synbiotic and *B. coagulans* supplementations were most effective in protecting goblet cells and mucin production, with GBRS also demonstrating a considerable effect compared to the DSS-control (Figure 5.4). A recent study (392) has demonstrated the ability of a *Bacillus* probiotic to upregulate the expression of TJ proteins in colitic mice, in line with these observations with *B. coagulans*.

The barrier integrity protection efficiency of GBRS supplementation could also be correlated to the ability of its RS component to induce SCFA production that in turn nourishes the colonic mucosa (77). The results of the current study suggest that a synergy between *B. coagulans* and GBRS combination imparted a substantial protection and/or maintenance of epithelial integrity in DSS-induced mice. While the efficacy of the synbiotic combination to stimulate TJ proteins and/or circumvent the TJ degradation by DSS needs further

investigation taken together the results support the ability of the prebiotic and probiotic combination to reinforce intestinal barrier integrity and help prevent the manifestation of IBD.

The breach of epithelial integrity in IBD also triggers aberrant inflammatory responses resulting in increased accumulation of pro-inflammatory mediators and thus further exacerbating the inflammation cascade and tissue damage (91). *B. coagulans* alone, and in synbiotic combination, demonstrated excellent immunomodulatory and anti-inflammatory efficacy as evidenced by reduction in colonic pro-inflammatory cytokine levels of IL-1α, IL-1β, Il-6, IL-12, TNF-α, and IFN-γ in both PC and DC segments. The results were similar to our previous study (42) that demonstrated marked immunomodulatory effects of *B. coagulans* MTCC 5856 spores *in-vitro* with colonic cell cultures. Levels of IL-1β, IL-6 and TNF- α were reported to be elevated in IBD patients (118). These cytokines are mainly secreted by activated lamina propria antigen presenting cells (APC) in response to the inflammation. APC's are part of the mechanism that maintains intestinal immune tolerance in the steady state but also prevent inappropriate responses to components of the gut microbiota that contribute to pathology in IBD (559). TNF-α, plays a pivotal role in triggering the accumulation and activation of leukocytes in colitis and hence is an important therapeutic target (524). Blockade of IL-6 signalling with monoclonal antibodies was also reported to be effective in reducing chronic intestinal inflammation in a mouse model. This effect was associated with the activation of T cell apoptosis and the suppressed production of pro-inflammatory IFN-γ (525). In contrast to a previous study (77), using green dwarf banana flour, that reported no effect on colonic cytokines, in the current study there was a noticeable reduction in levels of IL-1β, IL-6 and IFN-γ as well as reduction in serum IL-12 levels detected with GBRS supplementation. However, the respective immune-regulatory effects were less pronounced compared to that with Synbiotic supplementation, while there was no effect on levels of colonic IL-1α, IL-12, TNF-α and serum IL-10. Furthermore, in serum, Synbiotic supplementation induced marked reduction in pro-inflammatory IL-1β while, concomitantly increasing anti-inflammatory IL-10 indicating a synergistic effect.

B. coagulans MTCC 5856 spores have been demonstrated to impart excellent immunomodulatory effects to colonic cells *in-vitro* in an inflammatory state (42). This observation highlights the potential for application of probiotics with substantial immunomodulatory capacity, in conjunction with prebiotic with average immune-regulating effect, to potentiate combined anti-inflammatory effects to mitigate the aberrant immune

responses in IBD. IL-10 plays a prominent role in counterbalancing Th1 and Th17 immune activity in IBD towards a Th2 response by downregulating antigen presentation and subsequent release of pro-inflammatory cytokines thereby attenuating mucosal inflammation (527). IL-10 deficiency has been demonstrated to exacerbate colitis in the DSS-induced colitis model and IL-$10^{-/-}$ knockout mice have been shown to develop spontaneous colitis (560). Moreover, IL-10 administration has been determined to ameliorate colitis in mice by suppressing intestinal inflammation and reducing pro-inflammatory cytokine production (561). The anti-inflammatory potential of the synbiotic supplementation in the current study therefore warrants its application to human IBD trials to confirm the ability to regulate the exacerbated immune responses.

The supplementations also affected other indicators of the inflammatory response. The *B. coagulans* and Synbiotic supplementations suppressed increased colonic inducible nitric oxide synthase (iNOS) activity. Th1 and Th17 cytokines upregulate the iNOS expression and production nitric oxide (NO) in IBD that causes oxidative stress related inflammation and tissue damage (528). Elevated levels of CRP has been determined in human IBD (531). When inflammation is triggered, circulating IL-6 (partly induced by IL-1β and TNF-α) stimulate the production of CRP in the liver and subsequent release into the bloodstream (530). In the present study, elicited colonic IL-6 and serum CRP levels induced by DSS-induction were mitigated effectively by supplementation. Synbiotic, *B. coagulans* and GBRS supplementations displayed potent immune regulating efficacies to normalise the elevated serum CRP levels indicative of inflammation. Synbiotic was the most effective statistically ($P < 0.0001$) compared to DSS-control (Figure 5.6K). The combined immunomodulatory effect of *B. coagulans* and GBRS could be accounted for a potentiated synergistic efficacy of synbiotic supplementation in mitigating the pro-inflammatory cytokines in the current study. These findings indicate that the modulation of DSS-induced aberrant inflammatory responses by components of Synbiotic could potentially be owing to either a direct effect via suppression of pro-inflammatory cytokine, or to an indirect effect imparted by maintenance of epithelial integrity. Boost to the epithelial barrier functions would result in reduction of entry of foreign luminal antigens and thus lessen full activation of the innate immune system. There are therefore multiple mechanisms by which the action of synbiotic ingredient supplementation can address the underlying mechanisms that result in IBD pathology.

The microbiota-derived metabolites and SCFAs, which are the signatures of the gut microbiota and modulate immune activity in the gut, are important indicators of dysbiotic pattern in IBD (533, 534). The profiling for potential microbiota-derived untargeted metabolites in the present study, revealed distinct patterns between DSS-control and HC samples that are in agreement with previous reports (220, 221), that confirmed significant difference in faecal metabolic profiles IBD subjects compared to that of their healthy counterparts. The Synbiotic supplementation in the current study demonstrated excellent ability to modulate faecal metabolic profile of DSS-induced mice compared with that of the DSS-control. *B. coagulans* and GBRS supplementations alone could not mediate the same modulations in the metabolic profiles as observed by their synbiotic combination. This suggests the importance of application of the synbiotic strategy to achieve the most pronounced beneficial effects. A similar trend in effects was observed, in terms of SCFA profiles, where *B. coagulans* alone was not very effective in inducing SCFAs along the colon past the caecum. The Synbiotic and GBRS supplementations elicited elevated SCFA production along the entire length of colon. This indicated that, while the probiotic could induce increased fermentation in the caecum, the limiting factor post the caecum was the presence of fermentable substrate.

SCFAs made in the colon are active metabolites that function to reduce inflammatory mediators and increasing epithelial barrier function (562). The most abundant SCFAs in the colon are acetate, propionate and butyrate. These are produced by gut microbiota via fermentation of indigestible fibres. The concentrations of SCFAs vary along the length of the gut. The caecum and proximal colon show the highest levels that then decline towards distal colon segment (535). Moreover, in the caecum and colon 95% of SCFAs are absorbed by the colonocytes while only 5% are excreted in the faeces (563). Hence, determining the SCFA levels along entire length of colon including caecum is more instructive when assessing possible health effects than just measuring faecal SCFA levels. In this study the caecum showed the most abundant levels of SCFAs tested with all three supplementations confirming the findings of a previous study (536). However, this effect declined in the mucosal-associated and faecal samples with *B. coagulans* supplementation. Furthermore, a noticeable increase in SCFAs levels with GBRS and Synbiotic supplementations were observed along the entire length of the colon (caecal, mucosal-associated and faecal contents). The observation implied that *B. coagulans* alone could induce extra SCFA either directly, by metabolising available chow fibre, or indirectly by stimulation of metabolism of SCFA-producing gut bacteria. It is inferred that beyond the caecum fibre available for fermentation

could be limited. This conclusion is further supported by the ability of Synbiotic supplementation showing the higher levels of SCFAs production detected beyond caecum in mucosal-associated and faecal contents. Also, similar levels of SCFA produced by both GBRS alone and Synbiotic suggest the potential role of native microbiota, and possibly a limited role of the administered spore probiotic, in mediating greater SCFA levels. It is concluded that applying *B. coagulans*, along with GBRS could potentiate SCFAs production along the entire length of colon to mediate trophic beneficial effects in IBD. However clinical trials would be needed to determine if the mouse results also applied to the activity of the human gut microbiome.

There is good evidence that shows that reduced SCFAs concentrations, particularly butyrate, and its direct effect on microbial perturbations, results in defects in colonic barrier function and is associated with the related aberrant immune responses in IBD (551). *In-vitro* (538, 539) and *in-vivo* (540) studies have determined the effectiveness of butyrate in increasing epithelial integrity and mucus secretion. In the results of this study the considerable increase in butyrate levels by Synbiotic, GBRS and *B. coagulans* supplementation could be related to the positive effects observed on the histology of the colon, barrier integrity and reduction in disease severity in DSS-induced mice. Butyrate is the preferred energy source for colonocytes, and has the ability to regulate cytokines, thus showing protection against inflammation in UC and colorectal cancer (535). The improved expression of TJ proteins and mucus staining in goblet cells could be partially attributed to the elevated butyrate levels associated with Synbiotic supplementation in the present study. Acetate and propionate, that were found to elevate with Synbiotic supplementation along the entire colon length have also been found to benefit epithelial integrity via binding with certain metabolite-sensing G-protein-coupled receptors (such as GPR43, GPR109A) and modulating immune response (227, 239, 542).Valerate, that has been determined to stimulate intestinal growth and attenuate inflammatory pathogenesis in colitis (222), was increased by Synbiotic supplementation in this study. Therefore, a prebiotic component of synbiotic combination, that directly or indirectly influences SCFA production capacity of administered probiotic and gut beneficial microflora, is advantageous in modulating inflammation in IBD.

5.6 Conclusions

The research has highlighted a substantial efficacy of synbiotic supplementation carrying GBRS and *B. coagulans* spores in reducing the clinical manifestations and severity of DSS-induced colitis in a mouse model. The probiotic and prebiotic components complement each other to potentiate the beneficial effects. A substantial anti-inflammatory effect of the Synbiotic supplementation was generated by suppressing aberrant immune responses and colonic damage induced by DSS. The combination of the probiotic *B. coagulans* MTCC5856 and GBRS also improved the production of the metabolites and SCFAs that could similarly function to modulate the inflammatory parameters and ameliorate the disease severity. The observed synergistic functioning ameliorating or preventing the disease severity in DSS-induced mice model supports its further investigation for mitigating inflammation in human IBD. Furthermore, synergistic combinations of these synbiotic ingredients could be applied to develop novel shelf-stable foods targeted at improving gut health.

5.7 Supplementary data

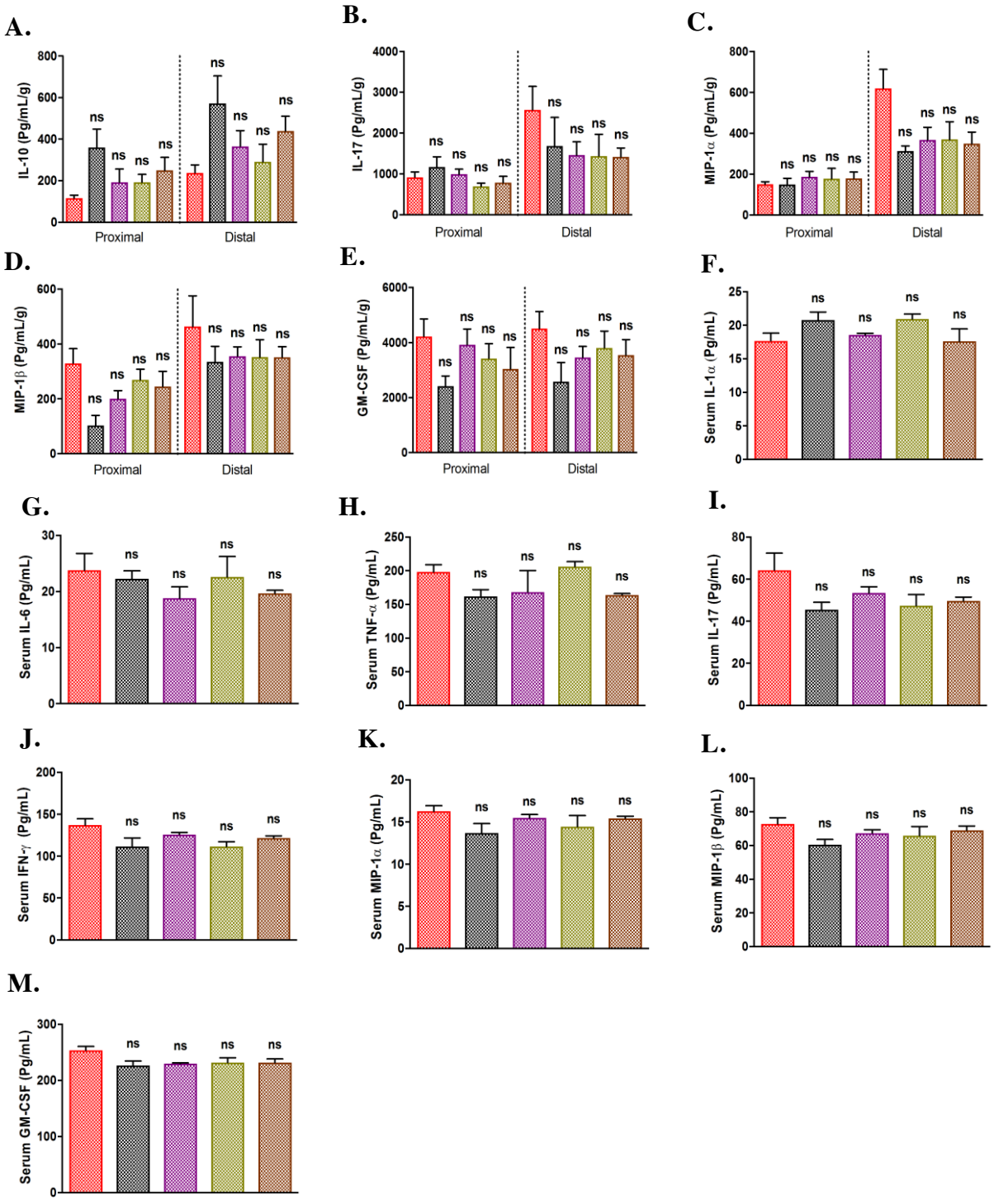


Figure SF5.1. Non-significant effect of *B. coagulans* spores, GBRS and synbiotic on immune markers in colon tissues and blood serum. Protein levels of cytokines including (**A**) IL-10 (**B**) IL-17, (**C**) MIP-1α, (**D**) MIP-1β, (**E**) GM-CSF in proximal and distal colon explants as well as cytokine levels of (**F**) IL-1α, (**G**) IL-6, (**H**) TNF-α, (**I**) IL-17, (**J**) IFN-γ, (**K**) MIP-1α, (**L**) MIP-1β, (**M**) GM-CSF in blood serum were analysed by Bio-plex. Statistical significance among groups evaluated by one-way ANOVA followed by Tukey's test. Non-significant (ns) vs. DSS-colitic group and data expressed as mean ± SEM (n = 3 per group).

Table ST5.1. Most significant compounds identified by OPLS-DA and SAM analysis in HC, DSS-control, *B. coagulans* (BC), GBRS and Synbiotic groups (*First 28 compounds are identified by SAM).

Compound name	InCHI Key	DSS-control (FC)	BC (FC)	HC (FC)	GBRS (FC)	Synbiotic (FC)	SAM (*P* value)
Uracil*	ISAKRJDGNUQOIC-UHFFFAOYSA-N	1.0273	0.93785	1.3456	0.92366	1.0277	0.0032787
Lyxosylamine*	RQBSUMJKSOSGJJ-AGQMPKSLSA-N	2.0177	1.8516	2.0713	1.9025	1.7637	0.004918
Linoleic acid*	OYHQOLUKZRVURQ-HZJYTTRNSA-N	0.91878	0.79878	1.0371	0.82761	0.77441	0.0059016
Glycerol*	PEDCQBHIVMGVHV-UHFFFAOYSA-N	1.1377	0.98485	0.20374	1.0278	1.0753	0.0062295
Hypoxanthine*	FDGQSTZJBFJUBT-UHFFFAOYSA-N	1.0261	0.90697	1.2559	0.92141	0.96043	0.0063934
Threonine*	AYFVYJQAPQTCCC-GBXIJSLDSA-N	1.1089	1.024	1.4418	1.0043	0.98399	0.0080328
Nicotinic acid*	PVNIIMVLHYAWGP-UHFFFAOYSA-N	0.58642	0.55666	0.78919	0.57325	0.54117	0.017213
Stearic acid*	QIQXTHQIDYTFRH-UHFFFAOYSA-N	1.1904	1.1258	0.089547	1.1692	1.1284	0.021967
Palmitic acid*	IPCSVZSSVZVIGE-UHFFFAOYSA-N	0.97012	0.97602	1.2616	1.0181	0.97063	0.022131
L-alanine*	MUBZPKHOEPUJKR-UHFFFAOYSA-N	1.4478	1.3039	1.2798	1.359	1.2537	0.024098
Glycine*	DHMQDGOQFOQNFH-UHFFFAOYSA-N	0.74802	0.82314	0.96068	0.83011	0.80656	0.030328
Myristic acid*	TUNFSRHWOTWDNC-UHFFFAOYSA-N	0.93638	0.98458	1.2561	1.0268	0.93831	0.038033
Oleic acid*	ZQPPMHVWECSIRJ-KTKRTIGZSA-N	0.90587	0.82736	1.1791	0.81412	0.81932	0.040656
Tagatose*	LKDRXBCSQODPBY-OEXCPVAWSA-N	0.76788	1.2614	1.0012	1.316	1.2004	0.042459
Altrose*	WQZGKKKJIJFFOK-VSOAQEOCSA-N	0.19196	0.81434	0.24376	0.82613	0.8301	0.060164
Glucose*	WQZGKKKJIJFFOK-GASJEMHNSA-N	0.76446	0.8646	0.54832	0.85449	1.0548	0.068689
Urea*	XSQUKJJJFZCRTK-UHFFFAOYSA-N	1.8628	2.1847	2.4975	2.2657	2.0866	0.072787
Talose*	WQZGKKKJIJFFOK-WHZQZERISA-N	0.32594	0.6913	0.39272	0.71034	0.77841	0.077869
Oxalic acid*	KZSNJWFQEVHDMF-BYPYZUCNSA-N	1.5115	1.326	2.0337	1.3618	1.2644	0.095246
Cellobiose2*	DLRVVLDZNNYCBX-ABXHMFFYSA-N	0.53979	0.7158	0.24935	0.48633	0.95198	0.096721
Cholic acid*	BHQCQFFYRZLCQQ-OELDTZBJSA-N	0.86044	0.76698	1.105	0.78972	0.75477	0.10689
Allo-inositol*	CDAISMWEOUEBRE-UHFFFAOYSA-N	0.35389	0.36113	0.47427	0.35022	0.40925	0.11574
Cellobiose1*	DLRVVLDZNNYCBX-ABXHMFFYSA-N	0.51337	0.66024	0.29171	0.461	0.95338	0.11574
Tyrosine*	OUYCCCASQSFEME-QMMMGPOBSA-N	0.33892	0.4797	0.34097	0.41096	0.68526	0.1359

4-Guanidinobutyric acid*	TUHVEAJXIMEOSA-UHFFFAOYSA-N	1.18	1.0594	0.89388	1.1051	1.0098	0.15246
Melibiose*	DLRVVLDZNNYCBX-ABXHMFFYSA-N	0.5164	0.65741	0.33806	0.47147	0.97777	0.16754
Lactose*	GUBGYTABKSRVRQ-DCSYEGIMSA-N	0.58297	0.71169	0.31237	0.52576	0.98415	0.17377
Allose*	WQZGKKKJIJFFOK-IVMDWMLBSA-N	0.53556	0.81397	0.66347	0.84108	0.79865	0.17557
Name (unidentified)	-	1.5577	1.3491	0.049086	1.4048	1.2883	
L-lactic acid	JVTAAEKCZFNVCJ-REOHCLBHSA-N	1.7802	1.5843	0.58119	1.633	1.5381	
Glycolic acid	AEMRFAOFKBGASW-UHFFFAOYSA-N	1.1274	0.99067	0.029675	1.0337	0.94309	
L-valine	KZSNJWFQEVHDMF-BYPYZUCNSA-N	0.678	0.63382	0.61791	0.64103	0.64384	
Benzoic acid	WPYMKLBDIGXBTP-UHFFFAOYSA-N	0.88099	0.83002	0.70659	0.86558	0.79141	
L-norleucine	LRQKBLKVPFOOQJ-YFKPBYRVSA-N	1.0204	0.92566	1.107	0.94936	0.90537	
Phosphoric acid	NBIIXXVUZAFLBC-UHFFFAOYSA-N	0.80246	0.94969	1.0798	0.77205	0.93011	
DL-isoleucine	AGPKZVBTJJNPAG-UHFFFAOYSA-N	0.6982	0.60584	0.73781	0.62697	0.59691	
L-proline	ONIBWKKTOPOVIA-BYPYZUCNSA-N	1.0582	0.93442	0.50926	0.95271	0.9057	
Succinic acid	KDYFGRWQOYBRFD-UHFFFAOYSA-N	1.4641	1.4847	1.5977	1.3243	1.4193	
Glyceric acid	RBNPOMFGQQGHHO-UWTATZPHSA-N	1.2087	1.1497	0.30975	1.1809	1.1151	
L-serine	MTCFGRXMJLQNBG-REOHCLBHSA-N	1.1618	1.0619	0.3067	1.072	1.0112	
Thymine	RWQNBRDOKXIBIV-UHFFFAOYSA-N	0.91938	1.0125	1.1993	0.9628	0.9986	
Malonic acid	OFOBLEOULBTSOW-UHFFFAOYSA-N	0.17821	0.2255	0.13648	0.18249	0.21455	
Iminodiacetic acid	NBZBKCUXIYYUSX-UHFFFAOYSA-N	0.35767	0.39998	0.2326	0.38002	0.44316	
Methionine	FFEARJCKVFRZRR-BYPYZUCNSA-N	0.22457	0.21064	0.22809	0.21886	0.30851	
Aspartic acid	CKLJMWTZIZZHCS-REOHCLBHSA-N	1.2721	1.1628	1.3098	1.1606	1.1147	
Phenylethylamine	BHHGXPLMPWCGHP-UHFFFAOYSA-N	0.59816	0.90911	0.41977	0.87604	0.92222	
Alpha ketoglutaric acid	KPGXRSRHYNQIFN-UHFFFAOYSA-N	0.67612	0.59347	0.36947	0.60824	0.56495	
Glutamic acid	WHUUTDBJXJRKMK-VKHMYHEASA-N	0.81057	0.82398	0.53876	0.74065	0.94431	

5-Aminovaleric acid	JJMDCOVWQOJGCB-UHFFFAOYSA-N	0.62455	0.843	0.12578	0.77829	0.84164	
Lyxose	SRBFZHDQGSBBOR-AGQMPKSLSA-N	0.47873	0.86784	0.61277	0.74513	0.82574	
Threitol	UNXHWFMMPAWVPI-QWWZWVQMSA-N	1.5245	1.3159	1.7783	1.371	1.2521	
Arabitol	HEBKCHPVOIAQTA-QWWZWVQMSA-N	1.5245	1.3159	1.7783	1.371	1.2521	
6-Deoxy-D-glucose	SHZGCJCMOBCMKK-GASJEMHNSA-N	1.1433	1.0515	1.2371	1.0452	1.0384	
Xylitol	HEBKCHPVOIAQTA-NGQZWQHPSA-N	0.76823	0.74181	0.88974	0.69986	0.71695	
Sorbose	LKDRXBCSQODPBY-AMVSKUEXSA-N	0.63081	1.0433	0.84948	1.0828	0.99622	
Allantoin	POJWUDADGALRAB-UHFFFAOYSA-N	1.6539	1.6207	1.9278	1.6881	1.5712	
Galactose	WQZGKKKJIJFFOK-SVZMEOIVSA-N	0.53147	0.80746	0.68121	0.83323	0.79426	
Tyramine	DZGWFCGJZKJUFP-UHFFFAOYSA-N	0.56357	0.58491	0.60375	0.52915	0.57178	
Lysine	KDXKERNSBIXSRK-YFKPBYRVSA-N	0.68847	0.6576	0.87344	0.65001	0.62596	
Sucrose	CZMRCDWAGMRECN-UGDNZRGBSA-N	0.71034	0.90616	0.89953	0.71426	0.91998	
Cholesterol	HVYWMOMLDIMFJA-DPAQBDIFSA-N	1.0646	0.96385	0.63693	0.96607	0.99383	

(International Chemical Identifiers (InChI) and standard InChI hashes (InChIKey); FC = Fold change)

Chapter 6

Efficacy of sugar cane fibre and probiotic spore synbiotic combination in attenuating chronic colonic inflammation in spontaneous colitic Winnie mice

6.1 Abstract

The efficacy of using prebiotic whole plant sugar cane fibre (PSCF) in combination with probiotic *Bacillus coagulans* MTCC5856 spores (*B. coagulans*) was investigated alone, and in synbiotic combination, for ameliorating chronic colitis in the spontaneous colitic Winnie (Muc2 mutant) mice model of IBD. Seven-week-old Winnie colitic mice were fed normal chow diet supplemented with either *B. coagulans*, PSCF or its synbiotic combination for 21 days. All three supplementations improved diarrheic stools, as well as prevented body weight loss. Synbiotic supplementation significantly ameliorated histological scores in both proximal ($p<0.0001$) and distal ($p=0.0443$) colon sections more effectively than either *B. coagulans* and PSCF alone. Moreover, Synbiotic supplementation substantially modulated the altered colonic and serum cytokine levels and lowered serum C-reactive protein (CRP) level compared to the unsupplemented Winnie-control. All three supplementations also resulted in noticeable modulation in the microbiota composition in caecal, mucosal-associated and faecal samples. While, PSCF favoured the abundance of *Akkermansia*, Synbiotic was effective in restabilising the depleted levels of *Prevotella* in Winnie colitic mice. Synbiotic was also significantly effective in elevating and normalising the levels of short-chain fatty acids along the length of the colon compared to that in unsupplemented Winnie-control mice. The augmented synbiotic effect could potentially be due to a combination of direct immune-modulating abilities of the components, their capability to improve epithelial integrity and/or modulation of the microbiota. Additionally, the symbiotic effect could also be a result of the increased levels of fermentation products elicit mitigation of inflammation. The beneficial effects in ameliorating the inflammation in spontaneous chronic colitic Winnie mice model of IBD warrants investigation of this synbiotic supplement in clinical trials.

6.2 Introduction

IBD, including UC and CD are chronic inflammatory conditions of the gastrointestinal tract and have multifactorial aetiologies (564). A three part pathophysiological circuit involving aberrant immune response, dysbiotic intestinal microbiota (and the associated metabolic pathways) and aberrant intestinal barrier function have been considered leading factors for causing ongoing chronic inflammation (19). The interaction of genetic, environmental and immunological factors is also believed to play prominent roles in the development and course of IBD. Additionally changes in the composition of the gut microflora have been associated with the pathogenesis of IBD (19, 138, 141, 143). The incidence of CD and UC is rising worldwide (493) despite the current medical treatments that focus primarily on immunosuppression (19). Treatments that hinder multiple factors involved in the recurrent inflammatory cascade would be a more pragmatic approach for mitigating the chronic inflammation associated with IBD.

Dietary interventions are being sought as adjuvant therapeutic treatments (9, 10, 23, 535). Dietary components can influence the composition of gut microbiota and the associated bacterial metabolic pathways, as well as interact with the immune system. In this context, a synergistic combination of prebiotic dietary fibre (DF) and probiotic bacteria are considered a potential approach to resolving the inflammatory cascade in the gut. Mechanistically this can be achieved by modifying the microbiota composition, the microbial metabolites, regulating secretion of immunomodulatory molecules and through protecting the colonic epithelial barrier (26, 28). The westernised diet, low in dietary fibres from fruits and vegetables, has also been linked to the surge in IBD incidence (9, 23). The biochemical complexity of DF is being regarded as a logical factor in influencing microbial complexity (52-54). Prebiotic fibres that are representative of whole plant vegetable and fruits, retain biochemical complexity and cell wall structures. Whole plant PSCF is made by grinding the dried, sucrose depleted cane. It therefore has 87% total dietary fibre content made up of both soluble and insoluble as well as rapid-and slow-fermentable fibres at ratios similar to that of whole plant foods (72, 73, 74). Moreover, to achieve potentiated synergistic beneficial outcomes, application of compatible probiotic that can metabolise these fibre fractions would be desirable.

B. coagulans can metabolise a variety of plant substrates rich in insoluble cell wall components (64, 71). *B. coagulans* spores are also GRAS affirmed and have been

demonstrated to confer substantial immunomodulatory, anti-inflammatory and anti-diarrhoeal effects (42, 48). It is therefore a candidate for synbiotic combination with prebiotic dietary fibres to augment its beneficial effects via SCFA production. Moreover, besides maintaining its stability during processing and storage of functional foods (43), *B. coagulans* spores have been shown *in-vitro* to survive during gastric transit and adhere to human colonic epithelial cells (42).

In the previous study (Chapter 4), pre-conditioning of mice PSCF and *B. coagulans* spores, prior to chemical induction of colitis using DSS, resulted in effective attenuation of acute colitis in DSS-colitic mice. The study clearly demonstrated that pre-conditioning of the gut with the synbiotic supplementation resulted in improvement in the severity of the clinical manifestations, histopathological features, immune markers and bacterial short-chain fatty acid production. The positive results may have been specific to the acute DSS model of colitis and changes in the microbiome could not be reliably measured due to interference of the DSS with PCR reaction. Therefore, the efficacy of the synbiotic combination carrying whole plant PSCF and *B. coagulans* in ameliorating chronic colitis was also tested using the well-established Winnie spontaneous chronic colitis mouse model.

Winnie mice are raised from a C57BL/6 background and demonstrate symptoms closely resembling to that of clinical IBD (565). Inflammation is evident by 6 weeks of age and progresses over time, leading in severe colitis, by the 16^{th} week (566). The chronic intestinal inflammation results from a primary intestinal epithelial defect conferred by a missense mutation in the Muc2 mucin gene (109, 566). Disruption of Muc2 biosynthesis initiates depletion of mucus layers, increasing intestinal permeability and leading to increased vulnerability to luminal antigens (109). The decrease in Muc2 production and secretion in active UC patients (567) and reduced expression of Muc2 in CD (568) are comparable to the variation in Winnie Muc2 aberration (565). Moreover, colitis in Winnie is chronic with periods of remission and relapse similar to human IBD (569). The inflammation in the distal region of the Winnie colon shows histopathological features of crypt elongation, neutrophilic infiltrates, goblet cell loss, crypt abscess formation, limited mucus secretion and focal epithelial erosions with an ulcerative colitis-type phenotype (109). Moreover, microbial gut dysbiosis and disruption in metabolomic profile in Winnie has been recently confirmed to be comparable to that in clinical IBD (217). Winnie mice have been shown to respond well to clinical drugs including MCC950 (501), glucocorticosteroids (570) and thiopurines (571,

572). However, the influence of dietary components such as probiotic or prebiotic on the disease pathogenesis in chronic spontaneous colitic Winnie mice is lacking.

It was hypothesised, based on the DSS model results (Chapter 4), that synergism between the whole plant PSCF and *B. coagulans* MTCC 5856 (*B. coagulans*) spores, would confer beneficial effects in attenuating chronic inflammation in spontaneous colitic Winnie. This study therefore aimed to investigate the therapeutic efficacy of the supplementations, alone and in combination, in the Winnie mice model of IBD and further examine its underlying mechanisms. The observations of this study would be useful in appreciating the effects of dietary components on the health of chronic colitic Winnie mice. This aspect has not been previously reported for this spontaneous colitic model. The Winnie model also offered the additional advantage of being able to extract DNA to profile the microbiome without the interference from DSS. This interference prevented good DNA extraction and profiling of microbiome changes in the DSS induced colitis model.

6.3 Materials and methods

6.3.1 Probiotic Bacteria and Prebiotic Dietary Fibre

LactoSpore® (Sabinsa Corporation, East Windsor, NJ, USA) containing the probiotic strain *Bacillus coagulans* MTCC 5856 (6×10^9 spores/gm) was produced by Sami Labs Limited (Bangalore, India) and supplied by Sabinsa Corporation (Australia). Kfibre™, prebiotic whole plant sugar cane fibre (PSCF) was supplied by KFSU Pty Ltd., Queensland, Australia (Appendix I).

6.3.2 Animals

Thirty-two, six-week-old Winnie mice (homozygous Muc2 mutant; C57BL/6J background) of both sexes and eight, six-week-old C57BL/6J wild type (WT) were obtained from the University of Tasmania animal breeding facility and housed in a temperature-controlled environment with a 12-hour day/night light cycle. Individual body weights were assessed daily including over an initial acclimation period of 7 days. All mice had access to radiation-sterilised rodent feed pellets (Barastoc Rat and Mouse, Ridley AgProducts, Australia, Appendix III) and autoclaved tap water for drinking *ad libitum* during experiments. All animal experiments were approved by the Animal Ethics Committee of the University of Tasmania [ethics approval number: A0015840 (Appendix IV)] and conducted in accordance

with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (8th Edition 2013). All efforts were made to minimize animals' suffering and to reduce the number of animals used.

6.3.3 Study Design and Treatments

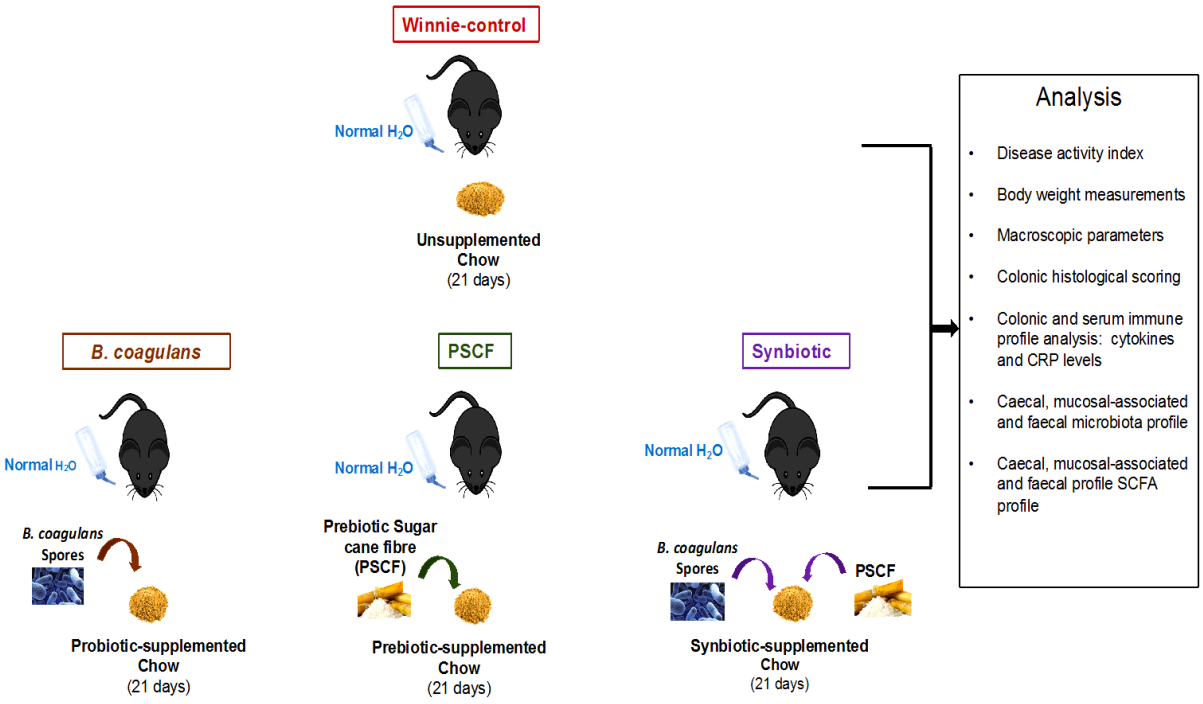


Figure 6.1. Experimental design of *in-vivo* feeding trial to analyse therapeutic efficacy of *B. coagulans* spores, PSCF and Synbiotic in chronic spontaneous colitis Winnie mice model. Colitic Winnie mice (n = 8 per group) that received normal drinking water were fed chow supplemented with either *B. coagulans* spores, PSCF or their Synbiotic combination for 21 days.

Following 1 week of acclimation, Winnie mice at 7 weeks of age were randomly allocated into the following 4 groups (n = 8 per group): (1) Winnie-control, (2) Probiotic *B. coagulans* MTCC 5856 (*B. coagulans*), (3) Whole plant prebiotic sugar cane fibre (PSCF) and (4) Synbiotic supplement. The experimental design of the mice feeding trial is illustrated in Figure 6.1. Mice in the Winnie-control group received 4g chow mash (standard chow pellet blended with water). The *B. coagulans* group received 4 g chow mash supplemented with probiotic *B. coagulans* MTCC 5856 spores (2×10^9 CFU/day/mouse). The PSCF group received 4g chow mash supplemented with Kfibre™ (200 mg/day/mouse). Synbiotic mice received 4 g chow, each supplemented with *B. coagulans* MTCC 5856 spores (2×10^9 CFU/ day/mouse) and Kfibre (200 mg/day/mouse). C57BL/6J wild type (WT) mice were also fed

4g normal chow mash. The chow mash was prepared fresh each day. The mice were single-caged throughout the experiment to measure the defined daily intake of respective treatments from prepared chow mash. The mice were fed these treatments for 21 days. Mice were sacrificed on day 22 by CO_2 asphyxiation.

6.3.4 Clinical Scoring and Histological Analysis

Body weight and disease activity index (DAI) was determined daily in all Winnie mice by scoring for changes in body weight, hemoccult reactivity, presence of gross blood in stool or at the anus and stool consistency, throughout the experiment, as detailed previously (501). DAI was determined by combining the scores from two parameters - stool consistency and presence of blood in the stool. These parameters were graded according to the scoring system as described previously in Chapter 4 (Section 4.3.4). Body weights were calculated for each animal throughout the experiments and weight calculated as percent weight change to the weight immediately before administration of supplemented chow (day 0). Faecal samples from Winnie and WT mice were collected on day 21 for short-chain fatty acid (SCFAs) and microbiota analyses. During faecal sample collection, mice were placed in cages with no bedding for approximately 2 hours to avoid contamination. At least 4 pellets (200-400 mg) were collected fresh from each mouse and transferred to microcentrifuge tubes using sterile forceps before storage at -80°C.

Following the sacrifice of mice, the measurement of macroscopic markers of inflammation (colon length and weight, spleen weight), collection of mucosal-associated and caecal contents for SCFA and microbiota profiling, collection of colonic tissues for cytokine analyses and preparation of proximal and distal colonic sections for histological staining by haematoxylin and eosin (H&E) was performed as detailed previously in Chapter 4 (Section 4.3.4). Slides stained with H&E (n = 6 per group) were graded blindly for the severity of tissue damage at distal and proximal regions based on the previously described scoring system (501, 504). Briefly, frequency of inflammatory infiltrate graded 0-3, goblet cell loss graded 0-3, crypt architectural distortion graded 0-3, frequency of crypt abscess graded 0-3, crypt hyperplasia graded 0-3, muscle thickening (oedema) graded 0-3, ulceration graded 0-3. The histological inflammation score for each proximal and distal colon region was derived from the sum of the score for each aforementioned criterion. All images were captured on a Leica DM500 microscope using a Leica ICC50 W camera (Leica Microsystems, Wetzlar, Germany).

6.3.5 Tissue Explant Culture and Cytokine Measurements

The cytokine levels in colon tissues (n = 3) and serum (n = 3) were determined by immunoassay using a Bio-Plex Pro Mouse cytokine 23-plex kit (Bio-Rad #M60009RDPD, Bio-Rad Laboratories, Australia) following the manufacturer's instructions and concentrations analysed using a Bio-Plex 200 instrument (Bio-Rad) and Bioplex Manager software, version 6 (Bio-Rad Laboratories) respectively as detailed in Chapter 4 (Section 4.3.8) (552). For tissues, the cytokine levels were normalized by dividing the cytokine results (pg/mL) by the measured biopsy weight (g) and the cytokines are presented as pg/g of tissue.

6.3.6 Serum C-Reactive Protein Analysis

The levels of C-reactive protein (CRP) in serum from respective groups (n = 3 samples/group) were analysed using Mouse C-Reactive Protein/CRP Quantikine Elisa kit (MCRP00, R and D Systems, Australia) following the manufacturer's instructions. The results are expressed as μg/mL.

6.3.7 Volatile SCFA Analysis

GC-MS analysis of 100–150 mg fresh weight (stored at −80 °C) of caecal, mucosal-associated and faecal samples (n = 5 per group) each was conducted for volatile SCFA profiling following the method described previously (510, 552) in Chapter 4 (Section 4.3.11). The GC-MS analysis was performed by Dr. David J. Beale (CSIRO), Dr. Avinash V. Karpe (CSIRO) and Dr. Shakuntala V. Gondalia (Swinburne University of Technology). Data analysis and interpretation was performed by the PhD candidate.

6.3.8 Microbiota analysis by 16s rRNA high-throughput sequencing

The total DNA was extracted from caecal, mucosal-associated and faecal samples (n = 5 per group) of Winnie and WT mice using the QIAamp DNA Stool Mini Kit (Qiagen, Melbourne, VIC, Australia). The samples underwent high-throughput sequencing on the Illumina MiSeq platform at the Australian Genome Research Facility (University of Queensland, Brisbane, QLD, Australia). Polymerase chain reaction (PCR) amplicons spanning the 16S rRNA V1-V3 hypervariable region with 27F forward primer (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R reverse primer (5'-

GWATTACCGCGGCKGCTG-3') were sequenced. Paired-end reads were assembled by aligning the forward and reverse reads using PEAR1 (version 0.9.5). Primers were identified and trimmed. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) 4 USEARCH 2.3 (version 8.0.1623) and UPARSE software (573). Using USEARCH tools, sequences were quality filtered; full-length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtering using "rdp_gold" database as a reference (574). To obtain several reads in each Operational taxonomic units (OTUs), reads were mapped back to OTUs with a minimum identity of 97%. Using QIIME, taxonomy was assigned using Greengenes database5 (Version 13_8, Aug 2013) (575). Image analysis was performed in real time by the MiSeq Control Software (MCS) v2.6.2.1 and Real-Time Analysis (RTA) v1.18.54, running on the instrument computer. RTA performs real-time base calling on the MiSeq instrument computer. The Illumina bcl2fastq 2.20.0.422 pipeline was used to generate the sequence data (574, 575). 16S rRNA gene sequences were analysed using MEGAN6 (Community edition version) (576), Microbiome analyst (577) and QIIME. Statistical analysis of Bray-Curtis dissimilarities was calculated using the relative abundances of bacterial genera using Adonis function in R (version 3.2).

6.3.9 Statistical Analysis

All data are presented as means ± SEMs. The statistical analysis was performed using GraphPad Prism Software (Version 7.0) The data were evaluated using One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test to determine statistical differences between the groups against Winnie-control samples. For the analysis of DAI and body weight changes during the experimental period, Two-way ANOVA was used followed by Tukey's post-hoc test, setting treatment and the time as the variables. A P-value of < 0.05 was considered significant. To determine overall microbial variation in the five groups, a principal coordinate analysis (PCoA) was used with Bray-Curtis ecological indexing and Euclidean distances as the similarity measure and Ward's linkage as a clustering algorithm (510). Two bacterial alpha (α-) biodiversity indices were evaluated, i.e. the Inverse Simpson Index and the Shannon Index. for both indices, an increased value indicates greater diversity (578). The data were evaluated with one-way analysis of variance (ANOVA) and using Tukey's test for multiple comparisons with a statistical significance of $P < 0.05$. For comparative microbial analysis, a linear discriminant effect size (LEfSe) analysis was performed ($\alpha = 0.05$), logarithmic Linear Discriminant Analysis (LDA) score threshold = 1.0.

6.4 Results

6.4.1 Effects of B. coagulans, PSCF and Synbiotic supplementation on clinical manifestations in Winnie mice

DAI (stool consistency and blood in faeces) and body weight changes were evaluated to determine the efficacy of the treatments in reducing the severity of disease symptoms in Winnie chronic spontaneous colitic mice (Figure 6.2). Compared with the Winnie-control group, that showed severe colitis symptoms (loss of body weight and diarrheic/bloody faeces), supplementation with *B. coagulans*, PSCF and Synbiotic significantly reduced the DAI levels as well as prevented body weight loss throughout the experiment (Figure 6.2A). At the end of the experiment, DAI of Winnie-control group was significantly higher (4.38±0.2) compared with that of *B. coagulans* (1.38±0.2, -69%), PSCF (1.75±0.3, -60%) and Synbiotic (1.37±0.3, -69%). Noticeably, PSCF was most effective in reducing DAI as early as day 3 mainly owing to improvement in stool consistency in comparison with *B. coagulans* and Synbiotic. In contrast to Winnie-control mice on day 21 (Figure 6.2B), Winnie mice supplemented with *Bacillus*, PSCF and Synbiotic treatments recovered body weight loss by 73.89%, 33.23% and 49.79% respectively.

The macroscopic evaluation of colonic segments revealed the beneficial effects of all three supplementations used in the study, as evidenced by marked reduction in colon weight/body weight ratio (*B. coagulans,* 21.01±1.7; PSCF, 23.57±1.0 and Synbiotic, 19.79±1.2 mg/g) compared with Winnie-control group (32.29±1.2 mg/g) (Figure 6.2C). None of the supplementations were effective in reducing the spleen enlargement (Figure 6.2D) that is also associated with colonic inflammation (499). Synbiotic supplementation was also significantly effective in reducing the colon length shortening (9.5±0.4) compared to the shorter colon length of Winnie-control mice (8.4±0.2 cm) (Figure 6.1E-F). The above markers are directly correlated to the severity of colonic damage in experimental model of colitis (499, 501).

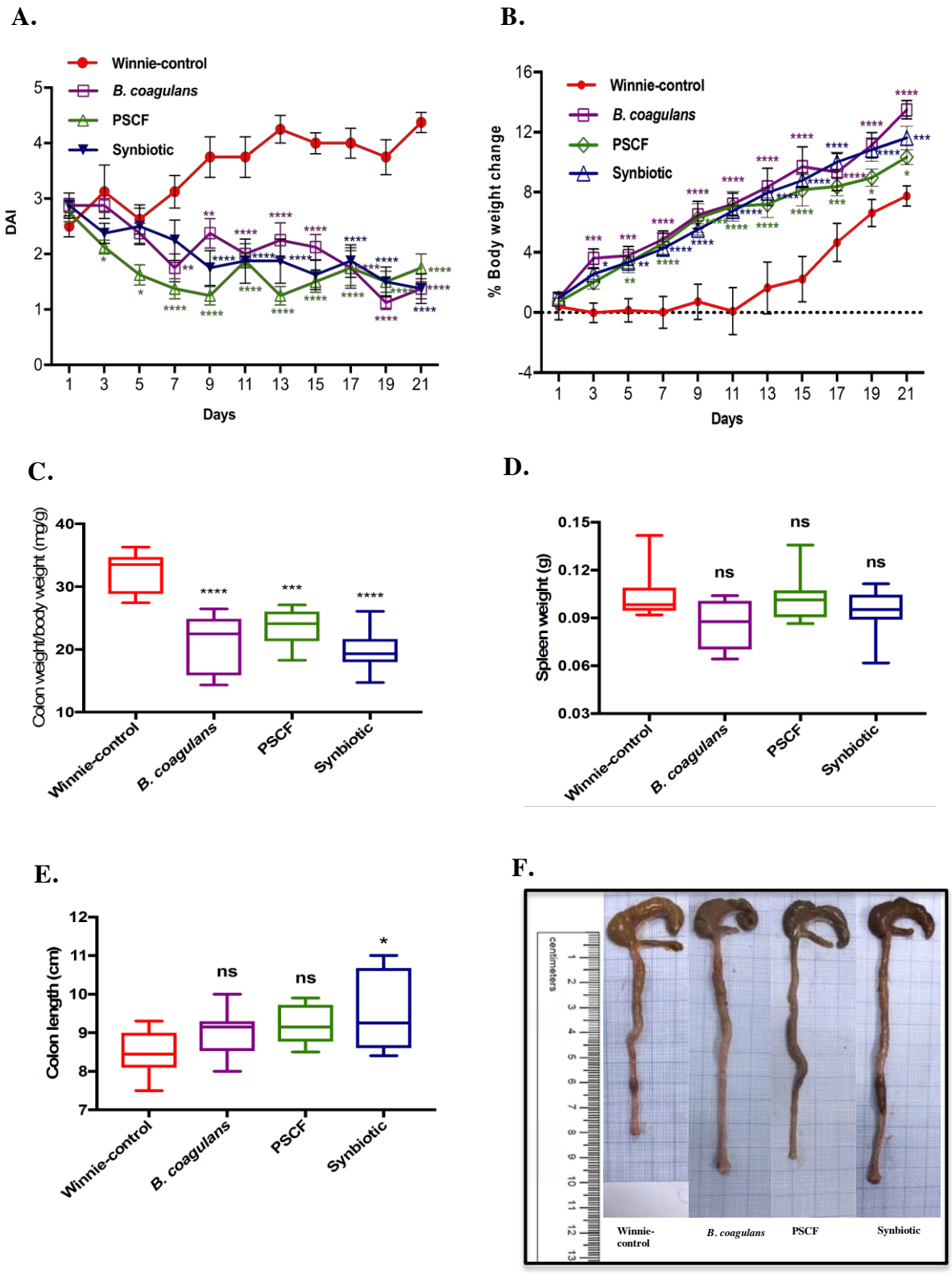


Figure 6.2. Effect of *B. coagulans* spores, PSCF and Synbiotic on clinical manifestations in chronic colitic Winnie mice. (**A**) Disease Activity Index (DAI), (**B**) % body weight change. Statistical significance among groups evaluated by two-way repeated-measures analysis of variance (ANOVA) followed by Tukey's test. **P* < 0.05, ***P* < 0.01, ****P*< 0.001, *****P*< 0.0001 vs. DSS-control group and data expressed as mean ± SEM (n = 8 per group). Colon weight/body weight ratio (**C**), Spleen weight (**D**), Colon length (**E**) and macroscopic appearance of colon (**F**). Data expressed as mean ± SEM (*n* = 8 per group), evaluated by one-way ANOVA followed by Tukey's Test. NS = non-significant, PSCF- Prebiotic sugar cane fibre.

6.4.2 Effects of B. coagulans, PSCF and Synbiotic supplementation on histological alterations in chronic colitic Winnie mice

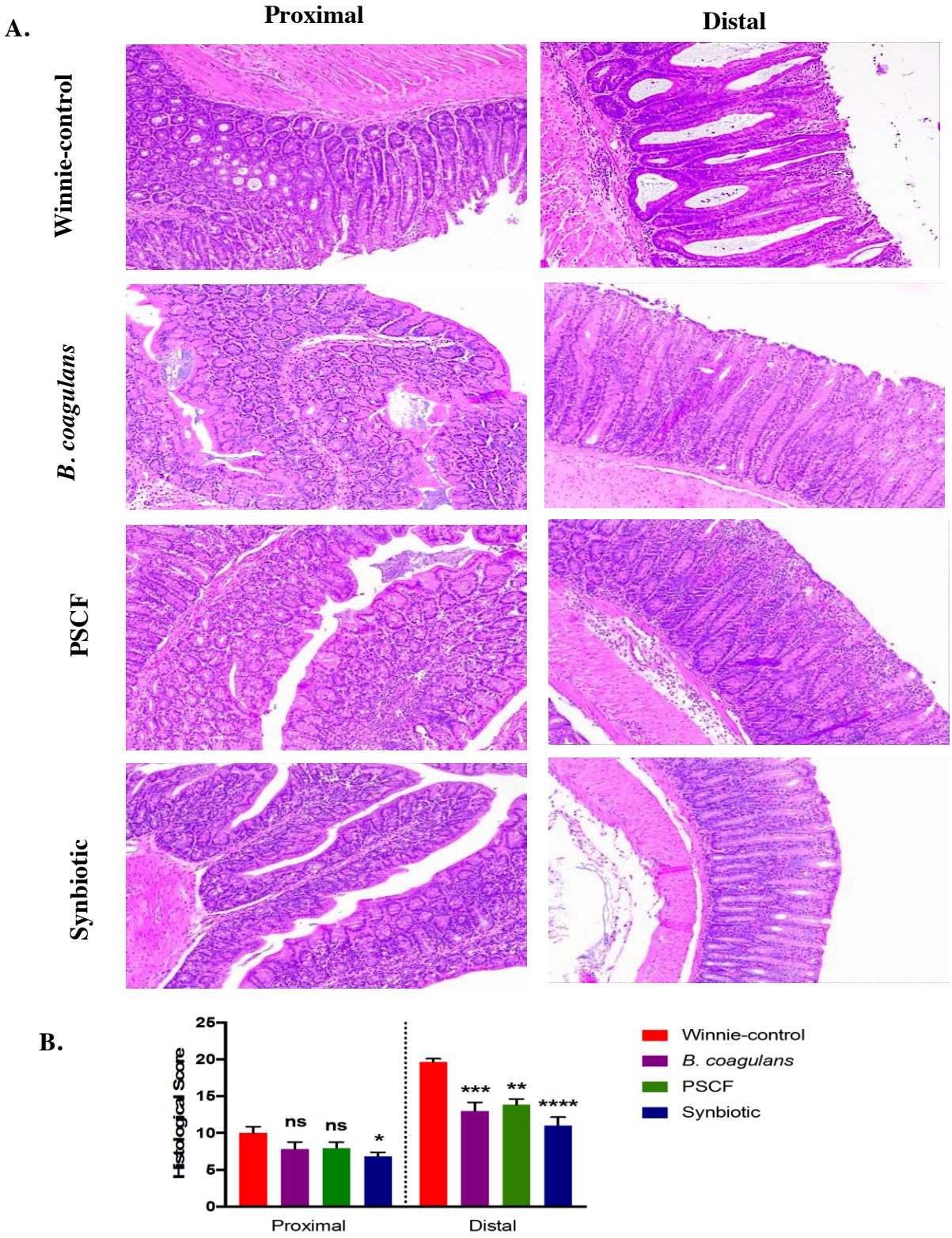


Figure 6.3. Effect of *B. coagulans* spores, PSCF and Synbiotic treatments on colon injury and inflammation in chronic colitic Winnie mice. (**A**) Histological images of proximal and distal colonic tissues stained with hematoxylin and eosin at 10x for each experimental group. (**B**) Histological score calculated after microscopic analyses of proximal and distal sections of the colon. Results expressed as mean ± SEM (n = 6 per group), evaluated by one-way ANOVA followed by Tukey's test (*P < 0.05, **P < 0.01, ****P < 0.0001).

Histological examination showed severe surface epithelial damage, crypt abscesses, crypt loss, distortion of crypt architecture, crypt hyperplasia and increased inflammatory infiltrate mostly affecting the distal colon (DC) section (Figure 6.3A) of Winnie-control compared with that of supplemented mice. Supplementation of Winnie with synbiotic (11, $P < 0.0001$), *B. coagulans* (13, $P = 0.0003$) and PSCF (13.8, $P = 0.0014$) displayed significant improvements in the histology of the colon, particularly in the distal section compared with the marked histological alterations score of 19.7 in untreated Winnie-control mice (Figure 6.3B). The comparative histological score for proximal colon (PC) was also statically lower ($P = 0.0443$) in Winnie supplemented with synbiotic (6.83) compared to that of Winnie-control (10). *B. coagulans* (7.83, $P = 0.2377$) and PSCF (8, $P = 0.3002$) alone were not statistically effective in reducing the histological score in PC, thus supporting the necessity of the synergistic Synbiotic combination to achieve consistent benefits.

6.4.3 Immunomodulatory effects of B. coagulans, PSCF, and Synbiotic supplementation on colonic immune markers

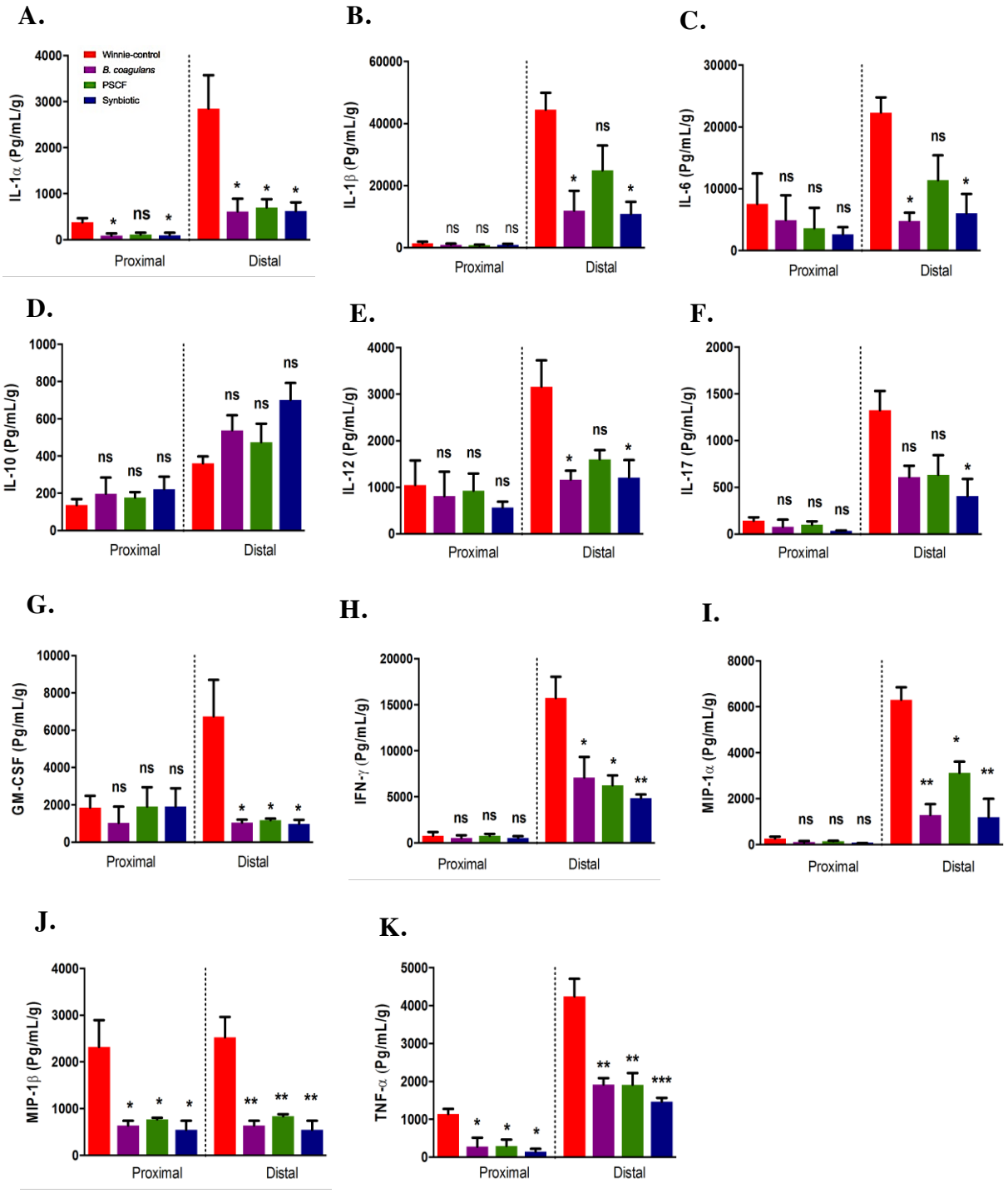


Figure 6.4. Effect of *B. coagulans* spores, PSCF and Synbiotic on immune markers in colon tissues. Protein levels of cytokines including (**A**) IL-1α, (**B**) IL-1β, (**C**) IL-6, (**D**) IL-10, (**E**) IL-12, (**F**) Il-17, (**G**) GM-CSF, (**H**) IFN-γ, (**I**) MIP-1α, (**J**) MIP-1β and (**K**) TNF-α in proximal and distal colon explants were analysed by Bio-plex. Statistical significance among groups evaluated by one-way ANOVA followed by Tukey's test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, ns-non-significant vs. Winnie-control and data expressed as mean ± SEM (*n* = 3 per group).

Cytokine analysis of the colonic tissue explants was used to ascertain the intestinal immunomodulatory and anti-inflammatory effects of *B. coagulans*, PSCF and their synbiotic combinations showing beneficial effects on the altered immune responses in spontaneous chronic colitic Winnie mice (Figure 6.4). The mucosal explants isolated from the colon of the untreated Winnie-control group showed marked secretion of a number of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12, IL-17, GM-CSF, IFN-γ, TNF-α) and chemokines (MIP-1α, MIP-1β) and a drop in anti-inflammatory IL-10 levels in both PC and DC sections. Supplementation of Winnie with synbiotic markedly suppressed the level of the elevated pro-inflammatory mediators, particularly in the DC section compared with that of the *B. coagulans* and PSCF supplementations alone. In PC the *B. coagulans*, PSCF and Synbiotic groups showed significant reduction in levels of MIP-1β and TNF-α. While PSCF was effective in suppressing the levels of IL-1α ($P = 0.0242$), IFN-γ ($P = 0.0179$), GM-CSF ($P = 0.0168$), MIP-1α ($P = 0.0204$), MIP-1β ($P = 0.0054$), and TNF-α ($P = 0.0022$) in DC no substantial reduction was noted for secretions of IL-1β ($P = 0.1867$), IL-6 ($P = 0.1065$), IL-12 ($P = 0.0644$), or IL-17 ($P = 0.1044$). Synbiotic and *B. coagulans* alone were statistically equivalent in suppressing the secretions of IL-1α ($P = 0.0203$, 0.0198 respectively), IL-1β ($P = 0.0195$, 0.0229 respectively), IL-6 ($P = 0.0173$, 0.0116 respectively), IL-12 ($P=0.0225$, 0.0198 respectively), GM-CSF ($P = 0.0139$, 0.0148 respectively), MIP-1α ($P = 0.0012$, 0.0014 respectively), and MIP-1β ($P = 0.0020$, 0.0027 respectively) in DC. However, Synbiotic compared to *B. coagulans* supplementation was more potent in reducing the levels of IL-17 ($P = 0.0304$, 0.1044 respectively), IFN-γ ($P = 0.0084$, 0.0292 respectively), and TNF-α ($P = 0.0007$, 0.0023 respectively). Moreover, Synbiotic supplementation elevated the anti-inflammatory IL-10 level in DC, although this was not statistically significant ($P = 0.0668$).

6.4.4 Immunomodulatory effects of B. coagulans, PSCF, and Synbiotic supplementation on systemic immune markers

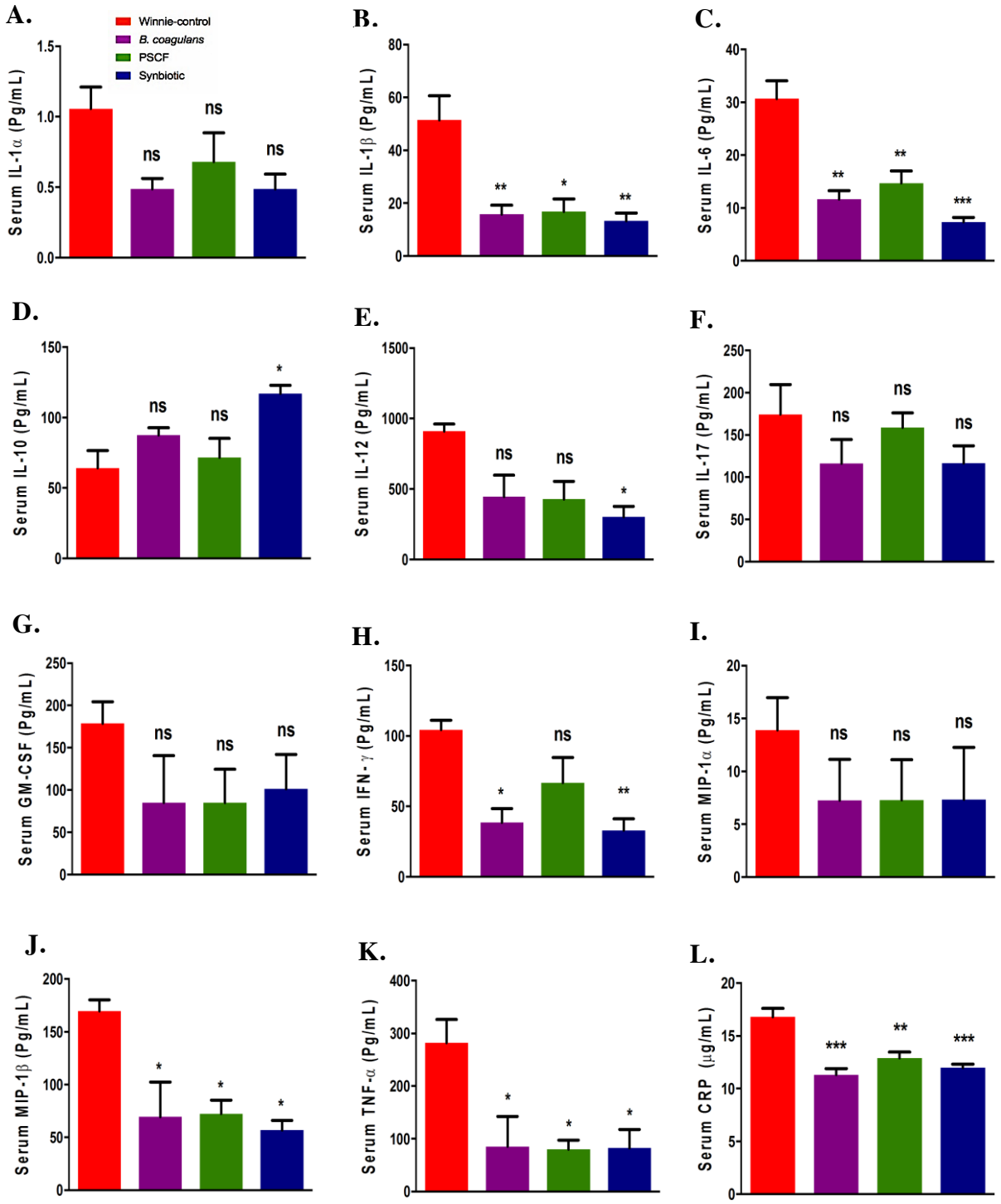


Figure 6.5. Effect of *B. coagulans* spores, PSCF and synbiotic on immune markers in serum. Protein levels of cytokines including (**A**) IL-1α, (**B**) IL-1β, (**C**) IL-6, (**D**) IL-10, (**E**) IL-12, (**F**) IL-17, (**G**) GM-CSF, (**H**) IFN-γ, (**I**) MIP-1α and (**J**) MIP-1β and (**K**) TNF-α in serum were analysed by Bio-plex. CRP levels in serum (**L**) measured by ELISA. Statistical significance among groups evaluated by one-way ANOVA followed by Tukey's test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, ns = non-significant vs. Winnie-control and data expressed as mean ± SEM (*n* = 3 per group).

None of the three supplementations were effective in reducing the serum levels of IL-1α, IL-17, GM-CSF and MIP-1α. However, substantial immune regulatory effects of the supplementations in Winnie were noted for other serum cytokines tested (Figure 6.5A-K). *B. coagulans* and PSCF supplementations alone suppressed the elevated serum levels of IL-1β (P = 0.0094, 0.0110 respectively), IL-6 (P = 0.0015, 0.0045 respectively), IFN-γ (P = 0.0156, 0.1739 respectively), MIP-1β (P=0.246, 0.0288 respectively) and TNF-α (P = 0.0384, 0.0338 respectively) in chronic spontaneous colitic Winnie. In addition to suppressing these cytokines, Synbiotic supplementation showed relatively more profound suppression in the levels of IL-6 (P = 0.0004) and IFN-γ (P = 0.0099) further supporting the existence of synergetic beneficial effects. Moreover, compared to Winnie-control, synbiotic supplementation significantly elevated the anti-inflammatory IL-10 levels in serum (P = 0.0233). It was more effective than *B. coagulans* (P = 0.4021) and PSCF (P = 0.9481) supplementations alone. Marked systemic immunomodulatory outcome effects of the supplementations in chronic colitic Winnie was also evidenced by the ability of *B. coagulans*, PSCF and its synbiotic combination to reduce the elevated CRP in the serum (11.32±0.58, 12.91±0.57 and 12±0.32 μg/mL respectively) compared to that in unsupplemented Winnie-controls (16.81±0.80 μg/mL) as depicted in Figure 6.5L. These observations, together with the prior DSS model results, further corroborate the substantial immunomodulatory and anti-inflammatory efficacies of the supplementations used in the study to reduce colonic and systemic inflammation in chronic colitis.

6.4.5 Effects of B. coagulans spores, PSCF and Synbiotic supplementation on microbial diversity in chronic colitic Winnie mice

Table 6.1. Comparison of Alpha (α) diversity indices evaluated in caecal, mucosal-associated and faecal samples obtained from wild-type (WT), Winnie-control, *B. coagulans* spores, PSCF and Synbiotic mice.

Sample site	Group	Shannon Index	Inverse Simpson Index
Caecal	WT	3.8 (±0.03)	11.7 (±0.25)
	Winnie-control	3.9 (±0.09)	12.3 (±0.45)
	B. coagulans	4.2 (±0.06)$^{\$}$	14.0 (±0.35)$^{\$\$,*}$
	PSCF	4.17 (±0.10)$^{\$}$	13.1 (±0.25)
	Synbiotic	4.2 (±0.06)$^{\$,*}$	14.6 (±0.45)$^{\$\$\$,**}$
Mucosal	WT	3.9 (±0.01)	11.85 (±0.29)
	Winnie-control	4.1 (±0.01)$^{\$}$	13.53 (±0.17)$^{\$\$}$
	B. coagulans	4.2 (±0.02)$^{\$\$}$	13.68 (±0.23)$^{\$\$\$}$
	PSCF	4.1 (±0.04)	13.47 (0.34)$^{\$\$}$
	Synbiotic	Undetermined	Undetermined
Faecal	WT	3.7 (±0.14)	10.5 (±0.34)
	Winnie-control	3.8 (±0.17)	10.7 (±0.27)
	B. coagulans	4.0 (±0.06)	12.23 (±0.37)$^{\$,*}$
	PSCF	3.9 (±0.04)	12.19 (±0.29)$^{\$,*}$
	Synbiotic	4.3 (±0.11)$^{\$\$,*}$	13.19 (±0.39)$^{\$\$\$,***}$

Statistical significance among groups evaluated by one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ Versus Winnie-control group and $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ versus WT group. Data expressed as mean ± SEM (n = 5 per group).

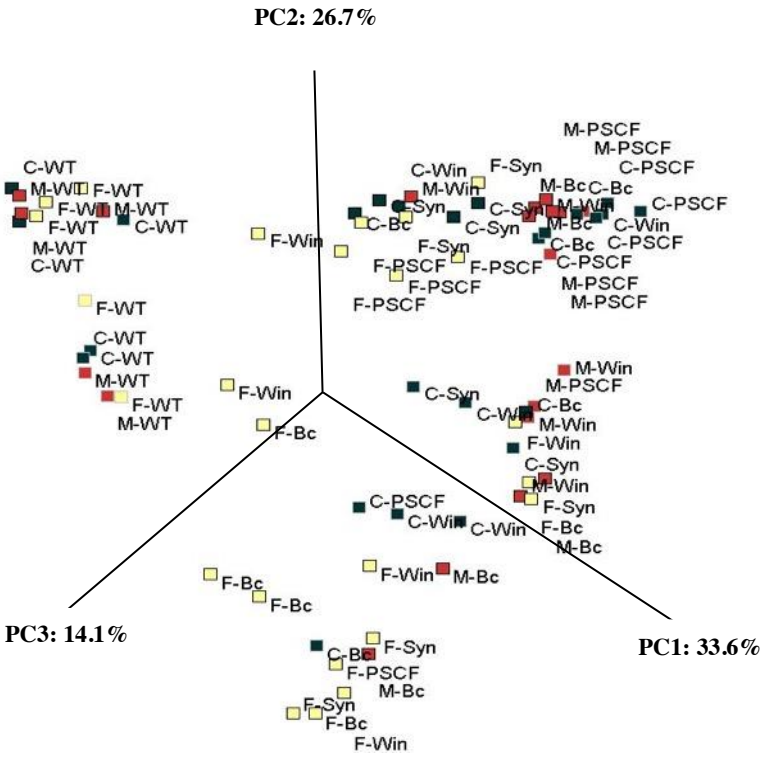
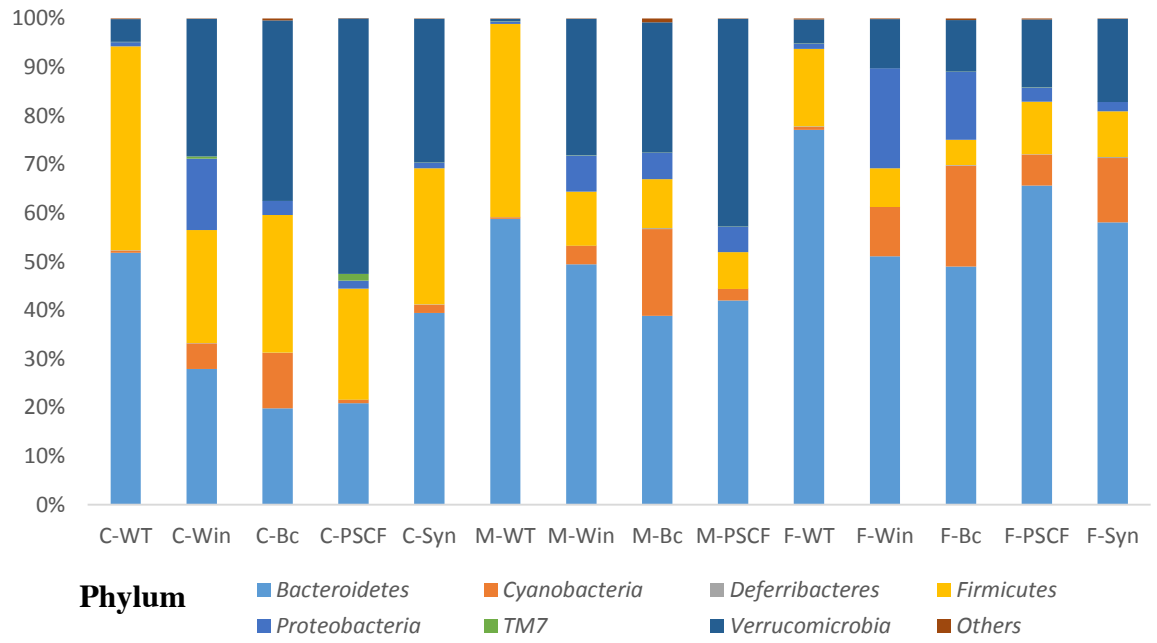


Figure 6.6. Principal component analysis (PCoA) plot based on Bray-Curtis distances calculated in caecal (C-), mucosal-associated (M-) and Faecal (F-) contents of wild-type (WT), Winnie-control (Win), *B. coagulans* (Bc) spores, PSCF and Synbiotic (Syn) groups; (n = 5 per group).

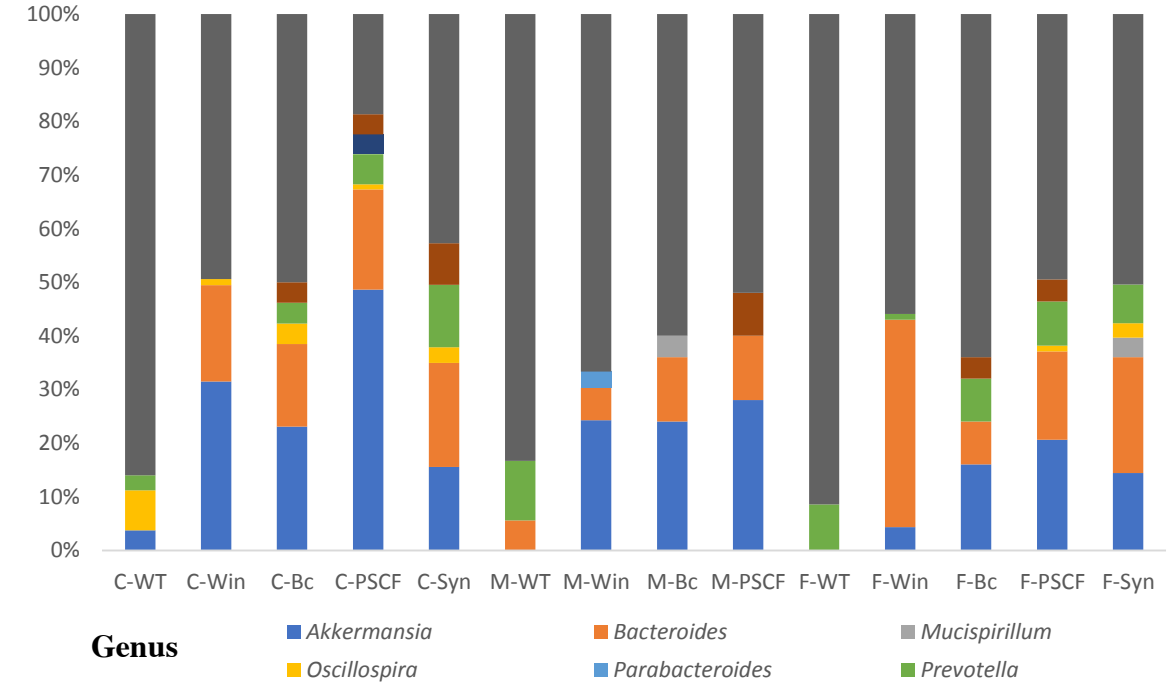
Taxonomic and functional profiles of 75 samples (n = 5 per group), including the caecal, mucosal-associated and faecal samples of WT and Winnie groups, were generated using the 16S rRNA gene sequencing-based method. In the experiment, 3 out of 5 synbiotic mucosal-associated samples collected did not generate the required 30,000 minimum sequencing raw read outputs hence the effect of synbiotic in modulating microbial diversity in mucosal-associated samples could not be determined. The effect of supplementation of diet with *B. coagulans*, PSCF and synbiotic in modulating microbial alpha and beta diversities in chronic colitic Winnie across caecal, mucosal-associated and faecal contents were assessed (Table 6.1). The Shannon index and inverse Simpson index, which are indices of alpha diversity, did not significantly differ between WT and Winnie-control mice samples except for in the mucosal-associated sample. The supplementations however, caused substantial increases in alpha diversity indices and the effect varied across the sample types. Compared with that of Winnie-control, synbiotic supplementation resulted in increased Shannon and Simpson indices in caecal and faecal samples. *B. coagulans* and PSCF supplementations alone however, resulted in significant modulation only in the Simpson index but had no effect on the Shannon-index. To evaluate beta diversity, PCoA plots of phylogeny with Bray-Curtis ecological indexing using ward clustering was used (Figure 6.6). The clustering showed distinct demarcation of samples (caecal, mucosal-associated and faecal) of WT group from that of Winnie groups (both supplemented and unsupplemented) with three distinct clusters at the operational taxonomic units (OTU) level among the 5 groups. This indicated clear differences in the microbial patterns between the healthy WT and the inflamed Winnie colitic mice. However, the microbial communities (irrespective of the sample types) of unsupplemented Winnie-control and supplemented Winnie groups were scattered, with no clear distinction between groups. This suggested high inter-individual variability among the Winnie-control and Winnie supplemented groups in terms of microbial diversity.

6.4.6 Effects of B. coagulans, PSCF and Synbiotic supplementation on microbial profile in chronic colitic Winnie mice

A.



B.



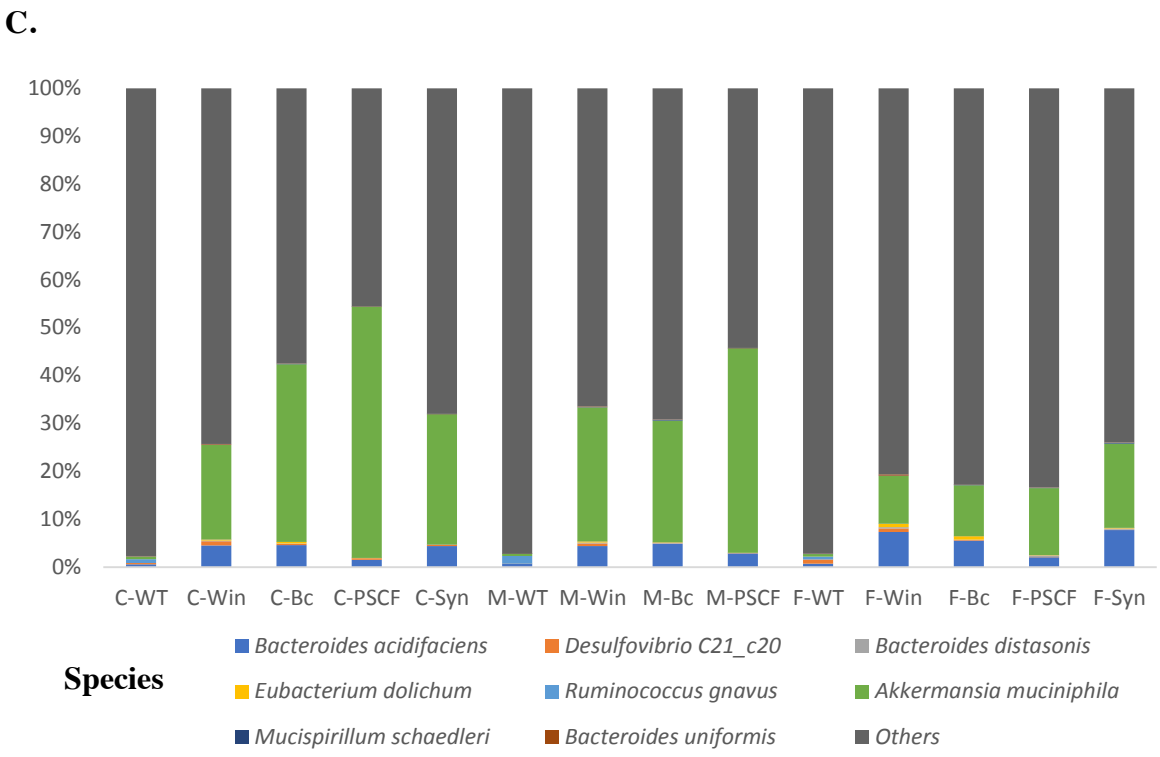


Figure 6.7. Relative abundances (%) of caecal (C)-, mucosal (M)- and Faecal (F)- associated microbiota at (**A**) phylum, (**B**) genus and (**C**) species level observed in wild-type (WT), Winnie-control (Win), *B. coagulans* (Bc) spores, PSCF and Synbiotic (Syn) groups; (n = 5 per group).

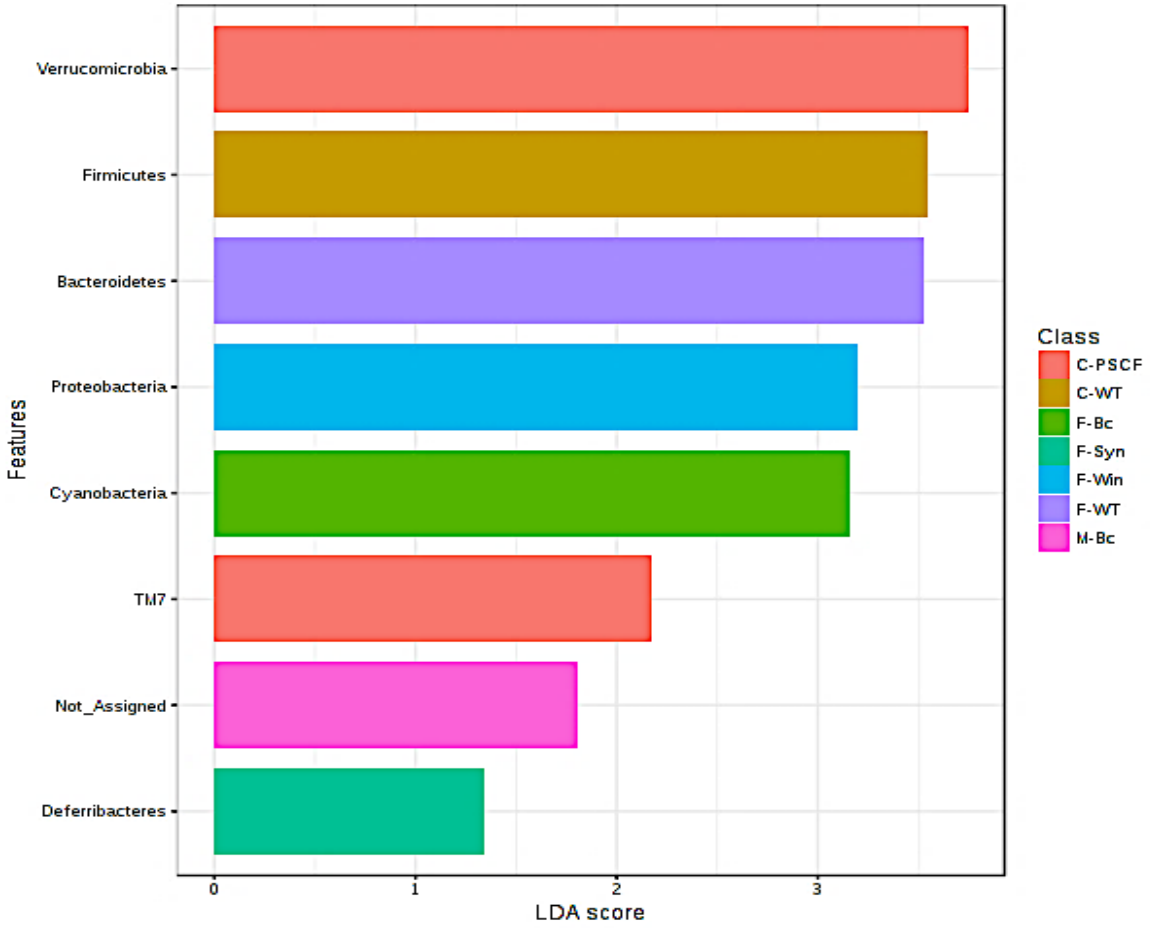
Figure 6.7A indicates the phylum-level changes in the caecal, mucosal-associated and faecal samples of WT and Winnie mice, which are dominated by Bacteroidetes and Firmicutes and moderately by *Verrucomicrobia*. Around 99% of the total microbial abundance was classified into seven major phyla (*Bacteroidetes*, *Cyanobacteria*, *Deferribacteres*, *Firmicutes*, *Proteobacteria*, *TM7* and *Verrucomicrobia*) in all sample types, while the rest were allocated as unclassified or others. Although Winnie mice shared most of the same phyla as healthy WT, levels of their abundance varied. While WT caecal and faecal samples showed 43% and 16% respectively of relative abundance of *Firmicutes*, their levels were reduced in Winnie-control to only 16% in caecal and 8% in faecal samples. Similarly, the phylum *Bacteroidetes* was also reduced in Winnie-control (19%) caecal samples compared to that of WT (54%). Though *B. coagulans* and PSCF supplementations resulted in elevation of *Firmicute* levels (28% and 22% respectively), no effect was observed for relative abundance of *Bacteroidetes* (19% and 20% respectively). Synbiotic supplementation however, increased *Firmicutes* (25%) and *Bacteroidetes* (36%) levels relative to that of Winnie-control in the caecum. Also, in faecal samples, Synbiotic supplementation was effective in inducing modulations in the levels of *Firmicutes* (25%) and *Bacteroidetes* (36%) compared with that in the Winnie-control (7.9% and 51% respectively). In contrast to WT, all

samples in Winnie groups showed increased abundance of *Verrucomicrobia*. As shown by LEfSe analysis (Figure 6.8A), among the Winnie experimental groups, PSCF supplementations caused substantial increase in *Verrucomicrobia* levels in caecal (52%) followed by in mucosal-associated (42%) and faecal (13%) samples. Similarly, compared to the spike in the level of *Proteobacteria* phylum in Winnie-control faecal samples (20.5%), Synbiotic suppressed the level (1.9%) similar to that in WT (1.1%). Among the other minor phyla, *TM7* (0.32% in Winnie-control mice) in caecum was suppressed by synbiotic (0.057%) and *B. coagulans* (0.022%) and were closer to the levels observed in WT (0.036%). PSCF however, increased TM7 levels in caecal samples (1.39%).

At the genus level, the distribution of microbial populations of Winnie-control mice was markedly different when compared to WT, in caecal, mucosal-associated and faecal microbiota (Figure 6.7B). While WT caecal samples showed the presence of *Oscillospira*, it was undetected in Winnie-Control. *B. coagulans* supplementation increased the abundance of *Oscillospira* in caecum, *Akkermansia* in faeces while modulating *Bacteroides* in faecal samples compared to that of Winnie-control. PSCF supplementation markedly enriched *Akkermansia* in caecal, faecal and mucosal-associated samples compared with that of in Winnie-control. Synbiotic supplementation in Winnie not only favoured the abundance of *Bacteroides* in faeces as revealed by LEfSe analysis (Figure 6.B), it was also observed to increase *Oscillospira* in caecal and faecal samples. While *Prevotella* showed its presence in caecal, mucosal-associated and faecal samples of WT, their levels were undetected in caecal and mucosal-associated samples of Winnie-control, while very low levels were detected in faecal samples. *B. coagulans*, PSCF and Synbiotic supplementations however, was able to induce appreciable increase in *Prevotella* levels in Winnie colitic mice. At the species level (Figure 6.7C), while WT samples showed the presence of *Ruminococcus gnavus* in all sample types, it was at very low levels in unsupplemented Winnie-control and supplemented Winnie groups. Compared to WT samples, Winnie samples showed increased prevalence of *Akkermansia muciniphila*. The ability of PSCF to substantially elevate the abundance of *Akkermansia muciniphila* in caecal samples was confirmed by LEfSe analysis (Figure 6.8B). PSCF also modulated their levels in mucosal-associated and faecal samples, while Synbiotic was effective in increasing their level in faecal samples the most. High levels of *Desulfovibrio* C21_c20 in faecal samples of WT and Winnie-control were greatly reduced with *B. coagulans*, PSCF and Synbiotic supplementations. Compared to WT, Winnie-control samples showed increased *Bacteroides uniformis*, while its level was suppressed by Synbiotic. *Bacteroides distasonis* remained undetected in the WT samples, while its presence was

detected in Winnie-control samples. The levels of these species were reduced marginally by Synbiotic supplementation in caecal samples while *B. coagulans* suppressing their level in faecal samples minorly. Additionally, *Eubacterium dolichum*, that were at undetectable levels in samples from WT mice, had a notable prevalence in the samples of Winnie-control but their levels were reduced by Synbiotic and PSCF supplementations.

A.



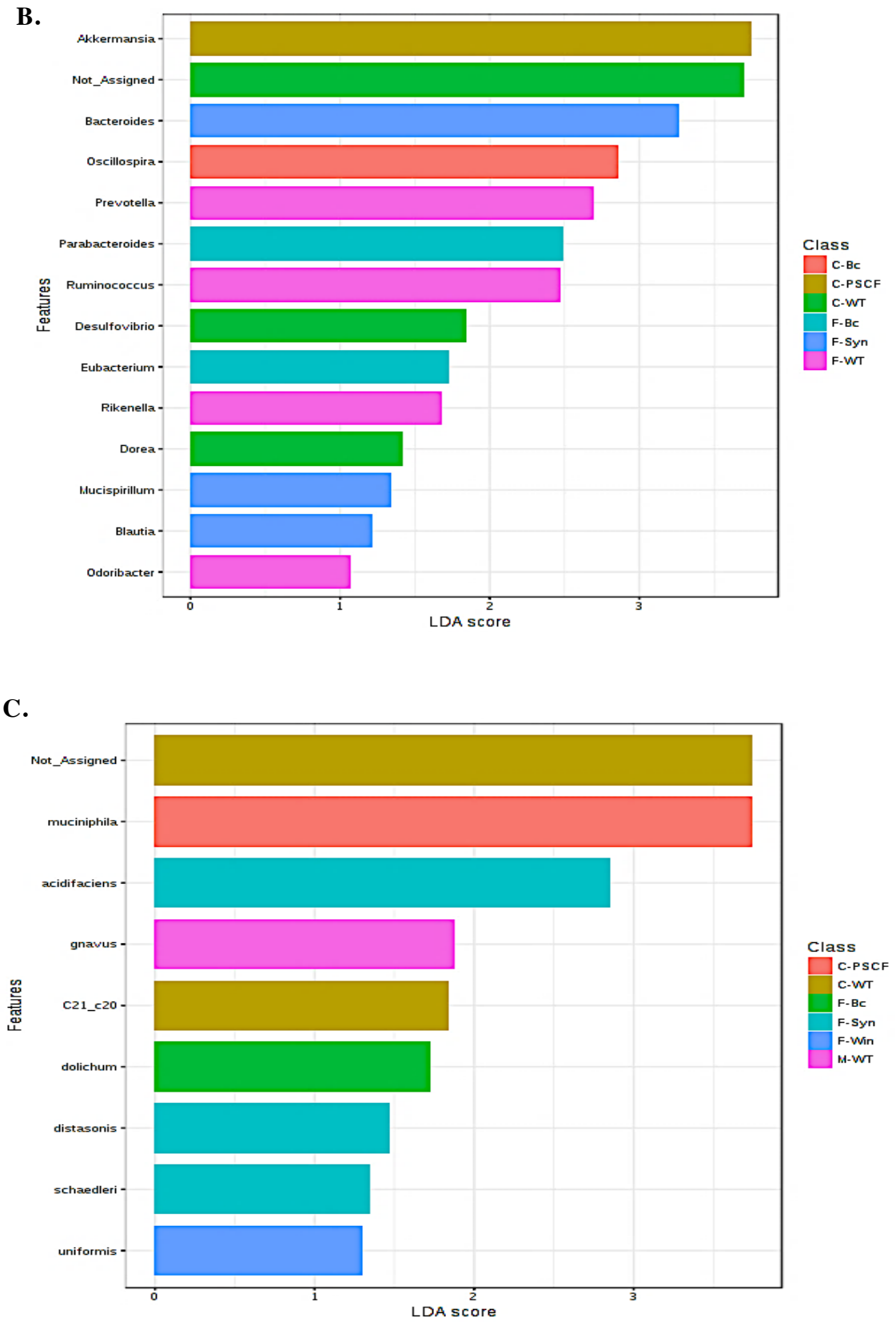


Figure 6.8. Biomarker analysis with Linear Discriminant Analysis (LDA) Effect Size (LEfSe) scoring plot using Kruskal-Wallis rank sum test (p = 0.01 and log LDA threshold cut-off value = 1.0)**.** Wild-type (WT), Winnie-control (Win), *B. coagulans* (Bc) spores, PSCF and synbiotic (Syn) groups at phylum (**A**), genus (**B**) and species (**C**) level. C-caecal, M-mucosal-associated, F-faecal.

6.4.7 Effects of B. coagulans, PSCF and Synbiotic supplementation on SCFA profile in chronic colitic Winnie mice

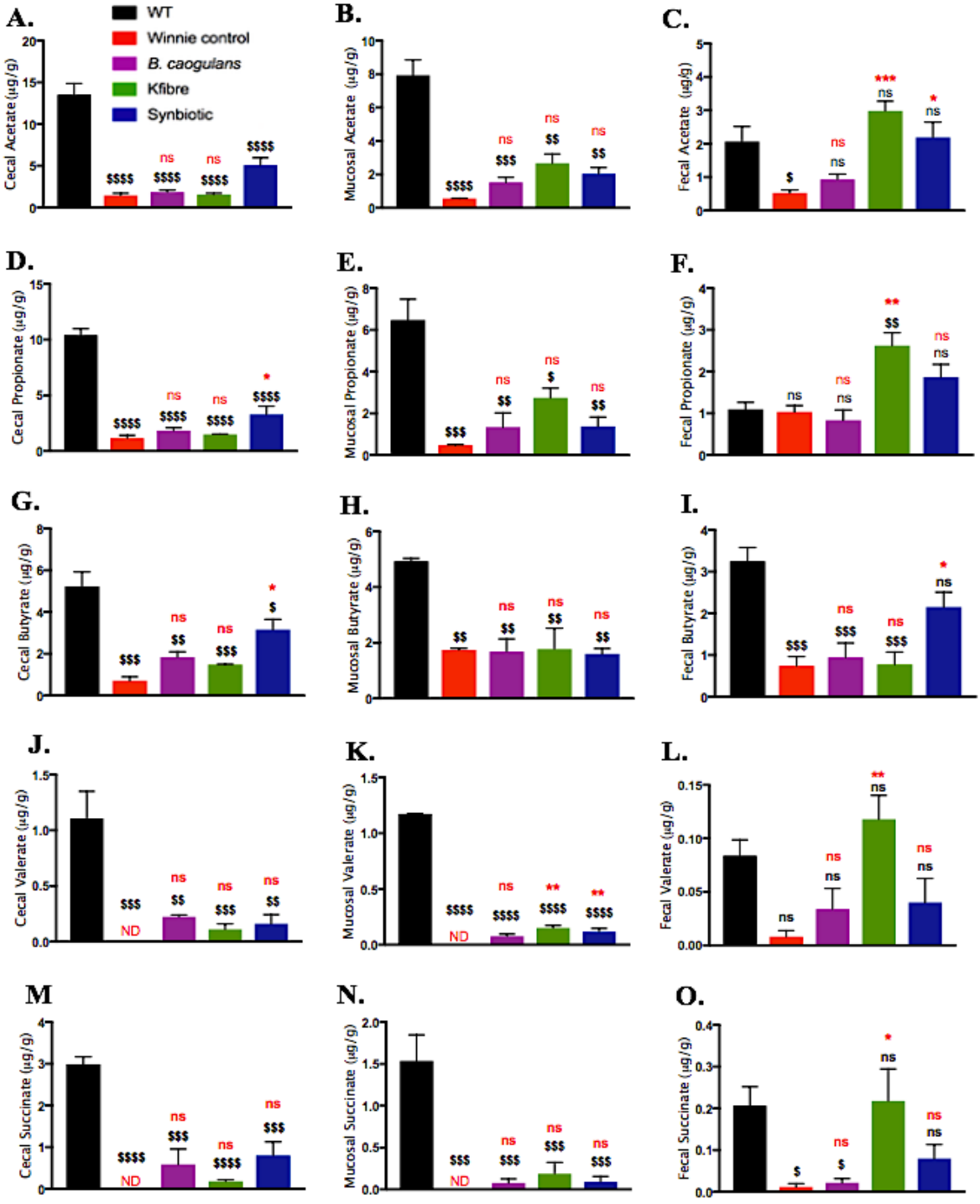


Figure 6.9. Effects of *B. coagulans* spores, PSCF and Synbiotic in modulating SCFA concentrations in caecal, mucosal-associated and faecal contents in Winnie vs. Wild-type (WT) mice. Statistical significance among groups evaluated by one-way ANOVA followed by Tukey's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns vs. Winnie-control group and $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$, $^{\$\$\$\$}P < 0.0001$, ns vs. WT group. Data expressed as mean ± SEM (n = 5 per group). NS = non-significant, ND = not detected.

As shown in Figure 6.9, feeding Winnie chronic colitic mice with PSCF, Synbiotic and to a lesser extent *B. coagulans* supplementations induced substantial modulations in the SCFA concentrations and their effects varied across caecal, mucosal-associated and faecal contents. Overall, higher concentrations of SCFA were noted in caecal contents than in mucosal-associated and faecal contents. Compared to the SCFAs concentration in the samples from WT mice, unsupplemented Winnie-control mice exhibited marked reductions in acetate, propionate and butyrate while, valerate and succinate were at undetectable levels. Although the supplementations were not statistically effective in normalising the whole SCFAs profile in range with that of WT, the supplementations substantially induced elevation in the tested SCFAs concentration relative to that of Winnie-control. While *B. coagulans* supplementation was ineffective in elevating the plummeted SCFAs levels in Winnie chronic colitic mice, PCSF supplementation alone showed ability to increase acetate (0.93±0.4 vs 2.98 μg/g), propionate (0.83±0.3 vs 2.62±0.3 μg/g), valerate (0.034±0.02 vs 0.118±0.02 μg/g) and succinate (0.022±0.01 vs 0.218±0.08μg/g) in faecal contents. Synbiotic supplementation was markedly effective in elevating the declined levels of acetate, propionate and butyrate in caecal and faecal contents indicating its synergetic functioning. In caecal contents, Synbiotic supplementation compared to the unsupplemented Winnie-control resulted in a significant increase in concentrations of acetate (5.07±0.9, 1.45±0.3 μg/g respectively), propionate (3.29±0.7, 1.15±0.3 μg/g respectively) and butyrate (3.16±0.5, 0.707±0.2 μg/g respectively). Moreover, Synbiotic supplementation in faecal contents, exhibited the excellent ability to not only increase the levels of acetate (2.19±0.4 μg/g), propionate (1.87±0.3 μg/g) and butyrate (2.15±0.4 μg/g) in Winnie colitic mice, the levels were equivalent to that of WT mice (2.06±0.4, 1.09±0.2, 3.25±0.3 μg/g respectively). Furthermore, only Synbiotic supplementation was effective in elevating butyrate levels along the entire length of colon (in caecal and faecal samples) relative to PSCF and *B. coagulans* supplementations alone. This finding indicates the prudence of application of synergetic Synbiotic components to provide elevated butyrate levels along the whole length of colon.

6.5 Discussion

In the current study, a dietary strategy involving the supplementations of *B. coagulans*, PSCF and their synbiotic combination was investigated in ameliorating chronic colitis in the spontaneous chronic colitic mice model Winnie. The findings indicate substantial efficacy of synbiotic supplementation in attenuating colonic inflammation in Winnie as evidenced by the improvement in clinical manifestations; colonic histopathology, colonic and systemic immune parameters. Additionally modulations of the microbiota and SCFAs fermentation products were induced by synbiotic supplementations more effectively and consistently than *B. coagulans* or PSCF alone.

In Winnie mice, spontaneous chronic colitis results from a primary intestinal epithelial defect conferred by a missense mutation in the Muc2 mucin gene, leading to symptoms of diarrhoea, ulcerations, rectal bleeding and weight loss similar to those in clinical IBD (565). In the current study, the gradual rise in DAI, body weight loss and excretion of diarrheic/bleeding faeces in Winnie was attenuated with *B. coagulans*, PSCF and Synbiotic supplementations compared to that in unsupplemented Winnie-control. The marked efficacy of PSCF in improving faecal consistency, leading to early improvements in DAI, may be associated with its high content of insoluble hemicellulose fractions. The hemicellulose fraction of plant fibre is known to possess large water-holding capacity and thus, contribute to regulating the faecal water content in colitic mice (288, 289). In a previous study (565), diarrhoea in Winnie mice, evidenced by a long size of the faecal mass moving from caecum to the anus, was associated with altered gastrointestinal transit times and disturbed colon motility compared with the wild-type (WT) mice. Similarly, alterations in the gastrointestinal transit times have been reported in IBD patients (579). PSCF supplementation has been confirmed to reduce diarrheic faeces resulting in improved DAI in DSS-induced colitic mice (552). *B. coagulans* have been reported in clinical studies to impart an anti-diarrheal effect (48). The beneficial effect imparted by synbiotic supplementation in the current study could be related to the synergistic actions between the bioactive components and supports its potential application in reducing diarrheal episodes in clinical IBD.

Synbiotic supplementation was also effective in escalating the improvement in DAI as well as macroscopic markers of inflammation (colon length and colon weight:body weight ratio), reinforcing evidence of ability to ameliorate disease severity and associated clinical manifestations in chronic colitic mice (Figure 6.2). Furthermore, the augmented beneficial

effects of the synbiotic application, compared to that of *B. coagulans* and PSCF alone, was evidenced by its ability to decrease the histological change scores in both proximal and distal colon sections of the Winnie mice (Figure 6.3B). The development of colitis in Winnie, with colonic histological damage mostly to the distal region, has been previously shown (109, 501). The ability of synbiotic treatment in reducing the colonic surface epithelial damage, by abating crypt loss, crypt abscesses, crypt hyperplasia, loss of goblet cells, submucosal edema and inflammatory infiltrate (Figure 6.3A), could be correlated with the improvement in the clinical manifestations in chronic colitic Winnie. Such synergetic beneficial outcomes of the *B. coagulans* and PSCF in synbiotic combination could be accounted for by the reinforcing of weakened colonic barrier integrity in chronic colitis.

Disruption in the colonic barrier integrity in IBD exacerbates dysregulated immune responses leading in an inflammation cascade and tissue damage (91). Disruption of Muc2 biosynthesis in Winnie colitic mice, is comparable to the reduction in production and secretion of Muc2 observed in human IBD (567, 568). This is known to trigger depletion of the mucus barrier, thus heightening intestinal permeability and increasing vulnerability to luminal antigens (109). The mucosal barrier dysfunction in the Winnie mice leads to colitis mediated by multiple cytokines (566, 580). The intestinal inflammation in IBD is marked by a Th1 and Th17-mediated responses with heightened expression of TNF-α, IFN-γ, IL-1β, IL-12, IL-6 and IL-17 (581). In the current study, unsupplemented Winnie-control colon segments were determined to secrete elevated levels of pro-inflammatory IL-1α, IL-1β, IL-6, IL-12, IL-17, GM-CSF, IFN-γ, MIP-1α, MIP-1β and TNF-α (Figure 6.4). This is in agreement with a recent study, that reported significantly increased levels of the pro-inflammatory cytokines in Winnie colon compared to that of wild-type (WT) mice (501). *B. coagulans* and PSCF treatment alone were able to reduce most of these elevated cytokines, however Synbiotic supplementation exhibited more consistent effects in suppressing the secretion levels of these pro-inflammatory cytokines in chronic colitic Winnie colon. IL-1β has been implicated to play a vital role in the pathogenesis of clinical IBD. In patients suffering from either acute or chronic gastrointestinal inflammation, increased levels of IL-1β cytokine have been reported (582). A number of clinical reports have confirmed the correlation of the increased IL-1β secretion from colonic tissues and macrophages of IBD patients with the severity of the disease (583-585). TNF- α is reported to incite a pro-inflammatory effect by inducing increased production of IL-6 and IL-1β (527). Similarly, increased levels of cytokines, such as IL-17, IFN- γ and IL-12, are observed in the mucosa of IBD patients (127). Furthermore, in the IBD-affected colonic

mucosa, accumulation of GM-CSF is associated with the delay of neutrophil apoptosis (586). Chemokines MIP-1α (also known as CCL3) and MIP-1β (also known as CCL-4), that induce pro-inflammatory cytokine production, are also observed to be highly expressed in IBD patients (587). The excellent ability of Synbiotic supplementation in the current study, was evidenced by its potentiated synergistic immune-modulatory efficacy in reducing the secretion levels of these pro-inflammatory cytokines and chemokines in the inflamed Winnie colonic tissue.

Increase in systemic pro-inflammatory cytokines are associated with IBD. At the systemic level, increased concentrations of IL-1β, IL-6, IFN- γ, and TNF-α were confirmed in IBD patients compared to healthy individuals (588). Such pro-inflammatory cytokines are secreted mainly by activated lamina propria antigen presenting cells in response to the inflammation (118). The capability of Synbiotic for imparting beneficial systemic anti-inflammatory effects was evidenced by its ability to suppress the levels of pro-inflammatory cytokines and chemokines in serum while also increasing anti-inflammatory IL-10. IL-10 plays a vital role in downregulating antigen presentation and subsequent release of proinflammatory cytokines, leading to attenuation of mucosal inflammation (527). *B. coagulans* spores have been confirmed to modulate IL-10 levels under *in-vitro* inflammatory conditions (42) and in humans (409). Furthermore, *B. coagulans*, PSCF and Synbiotic supplementations were effective in reducing the elevated serum CRP levels in Winnie further corroborating their immuno-modulatory capacities. High levels of CRP are reported in human IBD patients (531). CRP production in the liver and its release in the blood stream is stimulated by circulating IL-6 during inflammation (530). The marked ability of the supplementations in our study to reduce serum IL-6 and CRP levels supports their potential application in IBD to induce anti-inflammatory and immunomodulatory effects to hinder the inflammatory cascade. The amplified ability of Synbiotic for improving the overall pro-inflammatory profile of Winnie, could be attributed to either a direct immune-regulating effect of *B. coagulans* and PSCF, and/or due to their effect on improvement of colonic barrier integrity. Either of these effects could lead to a decrease in luminal antigen load and full activation of innate immune system. Our findings support the potential of synbiotic supplementations to be applied in clinical IBD to effectively mitigate the aberrant inflammatory responses and associated colonic damage.

Human clinical studies on the changes in microbiota associated with IBD are normally restricted to faecal sampling only. The dysbiosis associated with IBD however, may not be limited only to the faecal microbiota as the microbial numbers and composition vary along the gastrointestinal tract (139, 215). Mounting evidence has indicated that there is a distinction between the microbial dysbiotic pattern in different locations along the gastrointestinal tract of IBD patients, leading to the alterations in metabolic and immune responses (215, 589, 590). Therefore, the present study aimed to analyse site-specific profiles of microbiota and SCFA levels in caecal, mucosal-associated and faecal samples utilizing 16S rRNA gene sequencing and GC-MS for SCFA analysis. Besides the differences in the WT, unsupplemented Winnie-control and supplemented Winnie groups, this study confirmed microbial and SCFA shifts along the caecal, mucosal-associated and faecal samples. Although the indices of alpha-diversity did not significantly differ between the WT and Winnie-control samples, supplementation of chronic colitic Winnie with synbiotic was most effective in increasing the alpha diversity indices in caecal and faecal samples (Table 6.1). No significant difference in alpha indices between Winnie and WT faecal samples had been reported earlier by Robinson et al. (217). This may be attributed to the high inter-individual variability among Winnie mice suggesting, use of higher sample size for future analysis. Principal component analysis however, revealed a clear distinction in beta-diversity between WT and Winnie mice groups, further indicating the inflammatory status of the mice model (Figure 6.6). Notable differences in the caecal, mucosal-associated and faecal microbiota of WT and Winnie mice were evident at levels of bacterial taxonomical classification, including the phylum, genus and species. These observations agree with previous reports that confirmed distinct microbial patterns in faecal samples of inflamed Winnie and healthy WT mice (217). Although Winnie mice shared most of the same phylum as healthy WT mice, levels of their abundance were markedly different. In general, the abundance of members from Proteobacteria, Cyanobacteria and Verrucomicrobia in Winnie-control samples was greater, whereas reduced levels of Firmicutes and Bacteroidetes were evident compared to that of WT (Figure 6.7A).

Dysbiosis of gut microbial communities has been well recognized as one of the hallmarks of pathogenesis in IBD patients and animal models of colitis (138, 141, 214, 217, 591). Consistent with our results, Robinson et al. (217) also reported reduction in *Bacteroidetes* in Winnie-control compared with WT. Depletion of commensally associated bacteria, notably members of the phyla *Bacteroidetes* and *Firmicutes*, has been linked with IBD in several clinical reports (143, 214, 592). Compared with unsupplemented Winnie-control, Synbiotic supplementation elevated the levels of *Bacteroidetes* and *Firmicutes* in

caecal and faecal samples of chronic colitic Winnie. The genus *Prevotella*, belonging to phylum *Bacteroidetes*, was also significantly declined in the Winnie-control with only a low presence only in faecal samples, but its prevalence was enhanced by synbiotic supplementation in both the caecum and faeces. A high fibre diet has been linked to increased prevalence of *Prevotella* in healthy human subjects (593) and in African children consuming high-fibre, low-fat diets (10). *Prevotella* is a well-known dietary fibre fermenter for production of SCFAs (594). *Prevotella* species are also known to possess enzymes for degradation of an array of polysaccharides including cellulose, hemicellulose and xylans (10, 595). From this view, the increase in *Prevotella* levels in PSCF-supplemented Winnie could be correlated to the high content of plant cell wall fractions available for degradation. The ability of *B. coagulans* supplementation to also influence the abundance of *Prevotella* indicates a potential beneficial effect of the probiotic. The combined beneficial effect of increasing the prevalence of *Prevotella* and elevation of SCFA levels in Winnie by synbiotic supplementation suggests synergistic functioning. *B. uniformis* was detected in the biopsies of active UC patients (596). Synbiotic supplementation also decreased the level of *Bacteroides uniformis* (phylum *Bacteroidetes*) in faeces compared to that in the Winnie-control (Figure 6.7C).

The decreased prevalance of butyrate-producing Firmicutes is often associated with IBD (597, 598). Members of genus *Oscillospira* of *Firmicutes* are butyrate-producers (593). They were detected in WT but there was a complete absence in Winnie-control. The *Oscillospira* level was found to be severely decreased in IBD patients (597, 599). Synbiotic supplementation effectively recovered the altered levels of *Oscillospira* in caecum and faeces to that of WT. Synbiotic supplementation also influenced the level of *Blautia* of *Fimicutes* Phylum (Figure 6.8B) that has been reported in healthy Chilean individuals (593) and is one of the butyrate-producing bacteria in the human gut (214). In contrast to the previous IBD study in humans, that reported an increased prevalence of mucolytic *Rumicococcus gnavus* (112), Winnie mice showed complete depletion of *R. gnavus* (Figure 6.7B and 6.7C). None of the supplementations from this present research were able to restore its level. However, mucolytic activity of *R. gnavus* was shown *in-vitro*, to effectively degrade human Muc2 and porcine mucin (112). It also has an increased abundance in CD compared to that of UC in the intestinal mucosa of IBD patients. The increased population shift in *R. gnavus* was most apparent in non-inflamed histologically normal intestinal biopsies of IBD patients and there were lower levels of these mucolytic bacteria in inflamed UC tissue. It was hypothesized that less mucus in UC would be less favorable to this bacterium. Significant reduction in goblet

cells, reduced Muc2 expression and decreased mucin secretion in Winnie colitis model, similar to that reported in human UC (109), could be correlated to the decline of this mucolytic species in the current study.

Increased prevalence of *Proteobacteria* is considered a potential diagnostic signature of dysbiosis and risk of inflammation (600, 601). Relative to Winnie-control, Synbiotic was also more effective in reducing the abundance of phylum *Proteobacteria* in caecal and faecal samples compared with either *B. coagulans* or PSCF supplementation. *B. coagulans* and PSCF had no effect on *Proteobacteria* level in mucosal-associated samples. A significant increase in members of *Proteobacteria* phylum has been previously reported in faeces and in the caecal lymphoid patch of Winnie (217). Abundance of *Desulfovibrio C21_c20* species of *Proteobacteria* phylum were also reduced in Winnie supplemented with Synbiotic. The rates of hydrogen sulfide production were higher among the sulfate-reducing bacteria isolated from patients with UC compared to those in healthy volunteers (602). Species of genus *Desulfovibrio* are known to inhibit epithelial butyrate metabolism via release of hydrogen sulfide (603). These observations could be related to the decline in butyrate-producing bacteria and reduced butyrate production in inflamed colitic Winnie-control mice (Figure 6.9). The increased abundance of *Proteobacteria* in European children consuming a low-fibre, high-fat diet, compared to African children consuming high-fibre, low-fat diet, was reported by De Filippo et al. (10). In this context, the influence of Synbiotic in lessening the increased prevalence of *Proteobacteria* in Winnie mice in the current study, could be attributed to the high whole plant fibre content of PSCF.

High abundance of *Verrucomicrobia* members (*Akkermansia*) has been reported in healthy Chilean subjects (604) while its decreased abundance is noted in IBD patients (112, 178, 179). Interestingly, compared to that of WT, increased abundance of *Verrucomicrobia* was evident in all Winnie groups irrespective of supplementation/non-supplementation and the sample types. PSCF was most potent in inducing the bloom of *Verrucomicrobia* phylum, particularly in the caecum (Figure 6.7A and 6.8A). Members of genus *Akkermansia* and species *A. muciniphila* of *Verrucomicrobia* phylum were also elevated by PSCF supplementation in this research, specifically in the caecum. Additionally, in faecal samples all the three supplementations caused a moderate increase in *Akkermansia* genus level. The efficient colonisation of *A. muciniphila* in the caecum is attributed to the high mucin production (185). The increased ability of PSCF to induce growth of *Akkermansia* could be related to its polyphenolic content, as dietary polyphenols have been determined to promote

growth of *Akkermansia*, and was strongly correlated with the improvement of inflammation in DSS-induced colitis (605) and high-fat diet fed mice (606, 607). *A. muciniphila* uses mucin as nutrients (185). The increased prevalence of *Akkermansia* in Winnie mice, relative to that in WT mice, is surprising considering the less mucin being secreted, owing to the point mutation in Muc2 gene of Winnie (109). The bloom in the members of genus *Akkermansia* in Winnie could be partially related to its ability to metabolize the fatty acid hexadecenoic acid, which has been reported earlier to be heightened 2-3 fold compared with that in WT mice (217). Also, the aerotolerant ability of some species of *Akkermansia* confers resistance to the oxidative environment in the inflammatory colon (607, 608). In substantiation with our observation, increased abundance of *Akkermansia* was also reported in DSS mice model of colitis (180-182). Therefore there seems to be no clear consesus on the role of *Akkermansia* in chronic gut inflammation in IBD. In contrast however, *A. muciniphila* is known as a modulator for gut homeostasis (185) and is abundantly present in healthy human intestinal tract making up 1-4% of the bacterial population in the colon (183, 184). A recent study has demonstrated improvement in metabolic parameters in over-weight and obese human subjects by supplementation with *A. muciniphila* (609). Decline in its abundance is reported in human IBD patients suggesting its potential anti-inflammatory role (112, 178). A beneficial effect of *Akkermansia* on colitis however, is effected by its extracellular vesicles that were found to protect against DSS-induced colitis (186). Moreover, besides being able to degrade mucins, *Akkermansia* was also found to increase mucus layer thickness in prebiotic treated diet-induced obese mice, suggesting its potential ability to stimulate mucin synthesis (610). *Akkermansia* has additionally been demonstrated *in-vitro* to adhere to and restore the integrity of the epithelial cell layer, while no adherence was observed to human mucus thus, suggesting that the beneficial role of this bacterium in the gut is not exclusively associated with mucus layer physiology (608). Considering that the attenuation of colitic inflammatory parameters induced by PSCF and synbiotic supplementations was associated with a significant increase in *Akkermansia* in Winnie, a beneficial affect on gut inflammation is indicated. The ability of *B. coagulans* to enhance barrier integrity and mucus secretion (552), could be related to its influence on this mucosa-associated bacterium. While, PSCF increased abundance of minor phylum TM7 in the caecum, Synbiotic declined its level. High relative abundance of TM7 has been reported in the IBD patients (611) and IBD mice model (612). The efficacy of Synbiotic in reducing its level indicates a potential mechanism for its beneficial effect.

The Synbiotic supplement showed marked efficacy for modulating the altered SCFA production in chronic spontaneous colitic Winnie mice (Figure 6.9). SCFAs from caecal,

mucosal-associated and faecal samples were analysed to better understand the efficacy of *B. coagulans*, PSCF and their synbiotic combination in influencing the immune response and microbiota in Winnie colitic mice. Dysregulation in microbiota-derived SCFA production is often implicated with dysbiosis in IBD and therefore, has gathered considerable research interest (229). Of particular note are acetate, propionate and butyrate, each of which is likely to contribute to the host health (535). These SCFA, that are solely metabolized by gut bacteria from indigestible carbohydrates from fibre-rich diets, have been affirmed to attenuate disease severity in animal models of colitis (288) and in clinical UC (235). Consistent with the previous Winnie report (217), significant decline in the SCFA levels were detected in samples from unsupplemented Winnie-control mice compared to that in healthy WT mice. This has further confirmed the significant inflammatory and dysbiotic status of the Winnie colon. The altered SCFA production in Winnie could be associated with the decline in the abundance of SCFA producing bacteria belonging to genus *Oscillospira* and *Prevotella* as observed in Winnie-control group in the current study (Figure 6.7).

Reduced SCFA levels are an important indicator of dysbiosis in IBD. The consistently high ability of synbiotic supplementation to address the pathology caused by the Winnie mutation could be evidenced by its ability to elicit SCFA production in caecal and faecal samples thus, mediating a trophic effect along the entire length of the colon. This observation could potentially be correlated with the increased butyrate-producing *Oscillospira* genus with Synbiotic but was not detected in Winnie-control mice. Additionally, the ability of Synbiotic supplementation to promote the abundance of SCFA-producing *Prevotella* genus could be associated with the elevation in the SCFA levels. Butyrate is the preferred energy source for colonocytes and mediates regulation of cytokines further, imparting protection against inflammation in UC and colorectal cancer (535). Although, *B. coagulans* supplementation alone could not confer any substantial mediation in the SCFA production compared to Winnie-control, PSCF supplementation triggered elevations in the level of acetate and propionate in the faeces. However, this effect was not observed for the butyrate level. The propionate boosting effect of PSCF alone, could be correlated to its ability in inducing bloom in relative abundance of *Akkermansia* in Winnie (Figure 6.7). In *in-vitro* organoid testing, *A. muciniphila* was shown to induce substantial concentrations of propionate and acetate but not butyrate (613), in alignment with the observations of the current study. Its mucin degrading activity is known to mediate the production of propionate and acetate (614). Butyrate has been demonstrated in *in-vitro* (538, 539) and *in-vivo* (540) studies to increase epithelial integrity

consistent with the improvement in histological and immune-regulatory observations induced by Synbiotic in the present study.

The employment of a suitable probiotic bacteria targeted at metabolising the compatible prebiotic fibres to elevate SCFA production is a pragmatic synbiotic strategy towards resolving IBD inflammation. In the current study, relative to the individual supplementations, the synergistic synbiotic supplementation, not only induced increased levels of acetate, propionate and butyrate along the entire colon length, but the SCFA levels in the faecal samples were considerably restored to a level similar to that in WT mice. The inability of *B. coagulans* to modulate the SCFA production in this Winnie chronic colitic model, indicates two possible inferences: Firstly a possible lack of available fermentable fibre in normal chow diet to be directly metabolized by this probiotic and secondly, its compromised efficiency in promoting microbial growth of SCFA producers in chronic colitic inflamed Winnie colon. The ability of *B. coagulans* in inducing SCFA production in the caecum suggests the beneficial effects of pre-conditioning of the mice gut before colitis induction using DSS as previously demonstrated in Chapters 4 (552) and 5. This finding also indicates the need for early application of this probiotic spore in order to achieve the maximum benefit, as demonstrated in an *in-vitro* study in Chapter 3 (42). The excellent SCFA induction efficacy of *B. coagulans* in synbiotic combination with PSCF, suggests its ability to metabolise the fibre fractions to induce beneficial modulatory outcomes. The *B. coagulans* is known to ferment a variety of plant fibre including cranberry fibre (478), fenugreek seeds (477) and hemicellulose (71). Thus, the efficacy of *B. coagulans* in fermenting plant hemicellulose, such that present in whole plant PSCF, makes them an ideal bioactive combination for synbiotic application in conferring trophic effects of SCFAs in IBD along the entire colon length.

Acetate and propionate are also known to benefit the epithelial integrity via binding with certain metabolite-sensing G-protein-coupled receptors (such as GPR43, GPR109A) and modulating the immune response (227, 239, 542). Valerate, that has been determined to stimulate intestinal growth and attenuate inflammatory pathogenesis in colitis and cancer (222), was increased by PSCF supplementation. In addition to conferring benefits in the colon, SCFAs have also been confirmed to facilitate enhanced host metabolism and modulate the activity of the enteric nervous system (535), thus rendering extra-intestinal metabolic benefits. The excellent immuno-modulatory effects observed in the present study could possibly be correlated to high SCFA levels induced by Synbiotic supplementation in Winnie colitic mice owing to the synergistic combination. SCFA's are known to induce immune-modulation by

engaging with GPRs, leading to direct local and to systemic anti-inflammatory effects (227, 543) as evidenced by the improved cytokine profile in the current study. These observations merit the application of synergistic synbiotic combination to achieve potentiated benefits in resolving the inflammatory circuit in IBD.

6.6 Conclusions

The findings of this study highlight a significant efficacy of synbiotic probiotic and prebiotic supplementation in ameliorating the chronic colitis as evidenced by attenuation of spontaneous colitis in mice model of IBD. The results have demonstrated potentiated anti-inflammatory outcome effects of synbiotic treatment supplementation carrying whole-plant PSCF and *B*. *coagulans* spores by reducing clinical manifestations, colonic damage and inflammatory mediators while, modulating the gut microbiota and SCFA profiles of chronic colitic mice. The observations support the hypothesis that supplementation of whole plant PSCF and *B*. *coagulans* spores together produced a synergistic combination that augmented the beneficial outcome effects against the damage induced by chronic inflammation in spontaneous colitic Winnie mice. The study also underscored the application of Synbiotic in reducing the overall inflammation profile in this murine IBD model by targeting different mechanistic approaches to resolve the recurrent inflammatory cycle. The significant therapeutic effects of *B*. *coagulans* and PSCF in a synbiotic combination, evidenced in this study, warrants testing in human IBD trials to mitigate inflammation as an adjuvant therapy.

Chapter 7
Concluding Discussion

7.1 Summary of main findings

The main purpose of this research was to determine the efficacy of probiotic and prebiotic combinations of supplementary food ingredients for improving gut health. The work presented in this thesis has examined the effects of probiotic *Bacillus coagulans* MTCC 5856 spore alone and in a synbiotic combination with either whole plant prebiotic sugar cane fibre or green banana resistant starch flour on the disease outcomes of experimental acute and chronic colitis in murine models of IBD. It is worthwhile to note that while the effects were demonstrated in IBD pathogenesis, the anti-inflammatory effects could also be applied to low-grade inflammatory conditions including obesity, diabetes and related comorbidities involving gut inflammation. The ultimate goal of the investigation was to generate useful information to guide the development of functional synbiotic combinations that could potentiate beneficial effects for improving health outcomes through food. A specific outcome objective was to present a solution to the technical issues that limit application of conventionally used PB in shelf-stable functional foods owing to their low viability during industrial processing, storage and gastric transit (38, 348, 615). Using DF sources that more closely represented that of fruits and vegetables, with the biochemical complexity and cellular matrices of whole plant foods was a pragmatic choice due to their potential for influencing gut microbiota diversity for optimal wellbeing (52-54). This research hence, focussed on utilisation of effective functional ingredients: 1) probiotic *B. coagulans* spores that presented robustness in terms of survival in hostile conditions combined with substantial bioefficacy that could be applied in designing shelf-stable food products and 2) prebiotic fibres – whole plant PSCF and GBRS flour, both, derived from natural plant sources and prepared to retain nutritional biologically active components and more accurately represent natural whole plant foods. The prophylactic and therapeutic efficacies of these functional ingredients alone or in synbiotic combination were evaluated in ameliorating colitis in acute DSS-induced and chronic spontaneous Winnie colitic murine models.

The initial *in-vitro* screening in Chapter 3 successfully identified *B. coagulans* spores as an effective probiotic candidate. The key findings are illustrated in the Table 7.1. The high

survival of *B. coagulans* spores during simulated digestion supports their incorporation into wide variety of functional food matrices and pharmaceutical preparations without the need for encapsulation to preserve its bioactivity. Their substantial adherence to human colonic epithelial HT-29 and LS174T cells indicated their potential to interact with immune cells. Significantly more anti-inflammatory and immunomodulatory effects were exerted by *B. coagulans* spores when applied to non-inflamed and co-treated LPS-inflamed HT-29 cells than when applied to post-treated LPS-inflamed cells. This study therefore highlighted the importance of its application before (prophylactic agent) or during (therapeutic agent) the onset of inflammation to achieve optimum benefits. The excellent probiotic attributes evidenced in the *in-vitro* study thus confirmed its selection as an efficacious functional ingredient to be further tested for its anti-inflammatory potential in modulating gut health.

Table 7.1. Key findings of *in-vitro* screening analysis of *B. coagulans* spores presented in Chapter 3

Chapter 3 – Probiotic *Bacillus coagulans* MTCC 5856 spores exhibit excellent *in-vitro* functional efficacy in simulated gastric survival, mucosal adhesion and immunomodulation	
Screening parameter	**Key findings**
Simulated digestion	• High survival rate (92%) following 240 mins of simulated digestion
Adhesion to human colonic epithelial cells	• 86% adhesion to HT-29 cells • 81% adhesion to LS174T cells
Immunomodulatory capacity in non-inflamed and LPS-inflamed conditions in HT-29 cells	• Non-inflamed condition: ↑ IL-10 and ↓ IL-8 • LPS-induced co-treatment: ↑ IL-10 and ↓ IL-8 • LPS-induced post-treatment: IL-10 - not detected and ↓ IL-8 • Reduction in elevated IL-8 secretion more significant ($P = 0.018$) for co-treatment than post-treatment condition

The prophylactic and therapeutic efficacy of *B. coagulans* spore supplementation alone, and as a synbiotic combination, was further investigated *in-vivo* using murine acute and chronic models of colitis. Chapter 4 evaluated the prophylactic efficacy of dietary supplementation with *B. coagulans* spores and whole plant PSCF alone and as a synbiotic combination (PSCF-synbiotic) in influencing the onset and disease outcomes of DSS-induced acute colitis in C57BL/6J wild-type (WT) mice. Similarly, in Chapter 5, prophylactic efficacy of *B. coagulans* in synbiotic combination with a different whole plant DF source, the GBRS flour (GBRS-synbiotic) was investigated in attenuating the severity of DSS-induced acute colitis in WT mice. The key findings of the respective studies are listed in the Table 7.2. The

outcomes of the studies in Chapters 4 and 5 clearly supported the premise that pre-conditioning of the gut with synbiotic supplementations containing compatible probiotic and prebiotic fibres can be extremely beneficial in ameliorating the symptoms and severity of DSS-induced acute colitis in mice. This could potentially be attributed to the synergistic functioning of the probiotic with the respective prebiotic ingredients used in the studies. The probable mechanisms of synergy were via modulation of immune parameters, via SCFAs and by providing protection to the colonic epithelial integrity.

The potentiation of beneficial effects exerted by the synbiotic prompted the investigation into its efficacy in treating other gut inflammatory conditions. The study in Chapter 6 employed a spontaneous chronic colitic murine model, Winnie (Muc2 mutant), to evaluate the therapeutic efficacy of *B. coagulans*, PSCF and its synbiotic combination in ameliorating chronic colonic inflammation. The key findings are listed in Table 7.2. The results of this study also demonstrated marked efficacy of synbiotic supplementation carrying *B. coagulans* and PSCF in augmenting the attenuation of chronic inflammation. It therefore substantiated the conclusions derived from Chapters 4 and 5.

Table 7.2. Key findings of synbiotic efficacy of *in-vivo* analysis in Chapters 4, 5 and 6

Chapter 4 – Synbiotic supplementation containing whole plant sugar cane fibre and probiotic spores potentiates protective synergistic effects in mouse model of IBD	
Disease parameter	**Key findings**
DAI, % body weight loss and macroscopic markers	• Synbiotic most effective in reducing clinical manifestations (-72%), followed by PSCF (-53%) and *B. coagulans* (-47%) • PSCF imparted early improvement in stool consistency • All three supplementations effective in preventing body weight loss • Synbiotic most potent in improving all tested macroscopic markers
Histology and MPO activity	• Synbiotic significantly ↓ histology score and MPO activity in PC and DC • PSCF ineffective in ↓ histology score and MPO activity in PC
Alcian Blue staining for mucus	• Synbiotic, *B. coagulans* and PSCF prevented goblet cells and mucus production with synbiotic noted for highest Alcian blue staining intensity
Immunohistochemical detection of epithelial TJ proteins	• Synbiotic most efficacious in preserving the expressions of all the TJ proteins tested (ZO-1, Occludin and Claudin-1) • *B. coagulans* followed by PSCF also moderately preserved TJ proteins
Immunomodulatory effects on colonic and serum cytokine levels, iNOS activity and CRP level	• Consistently marked immunomodulatory effect exerted by synbiotic on colonic and serum cytokines • Differential effects observed with PSCF in PC and DC sections and no effect noted for colonic IL-12 and IFN-γ and serum IL-1β and IL-10 • *B. coagulans* failed to reduce serum IL-1β level

	• No effect on iNOS activity in PC with PSCF while, synbiotic and *B. coagulans* equally effective in both sections • Synbiotic significantly normalised serum CRP levels followed by *B. coagulans* and PSCF
Faecal metabolite analysis	• Synbiotic significantly efficacious in inducing modulations in the faecal metabolites
SCFA profile of caecal, mucosal-associated and faecal samples	• Highest concentration of SCFAs observed in caecum • *B. coagulans* lost ability of SCFA induction past caecum • Synbiotic most adequately ↑ SCFA levels along the colon length
Chapter 5 – Prebiotic green banana resistant starch and probiotic *Bacillus coagulans* spores synbiotic supplementation ameliorates gut inflammation in mouse model of IBD	
Disease parameter	**Key findings**
DAI, % body weight loss and macroscopic markers	• Synbiotic most effective in reducing DAI (-67%), followed by GBRS (-57%) and *B. coagulans* (-52%) • All three supplementations effective in preventing body weight loss • Synbiotic most significant in improving all tested macroscopic markers
Histology and MPO activity	• Synbiotic and *B. coagulans* followed by GBRS significantly ↓ histology score only in DC • GBRS ineffective in reducing MPO activity in PC
Alcian Blue staining for mucus	• Synbiotic and *B. coagulans* noted for equivalently marked improvement in Alcian blue staining intensity suggesting benefits to goblet cells and mucus production with PSCF showing moderate effect
Immunohistochemical detection of epithelial TJ proteins	• Synbiotic most potent in retaining the expressions of all the TJ proteins tested (ZO-1, Occludin and Claudin-1) • *B. coagulans* followed by GBRS also moderately preserved TJ proteins
Immunomodulatory effects on colonic and serum cytokine levels, iNOS activity and CRP level	• Consistently substantial immunoregulatory effect exerted by synbiotic on colonic and serum cytokines • Differential effects observed with GBRS in PC and DC sections and no effect noted for colonic IL-1α, IL-12 and TNF-α in DC and serum IL-1β and IL-10 • *B. coagulans* failed to reduce serum IL-1β level • GBRS ineffective in reducing iNOS activity in both colonic sections • Synbiotic effectively normalised serum CRP levels followed by *B. coagulans* and PSCF
Faecal metabolite analysis	• Synbiotic significantly induced modulations in the faecal metabolites
SCFA profile of caecal, mucosal-associated and faecal samples	• Highest concentration of SCFAs observed in caecum • *B. coagulans* lacked ability of eliciting SCFA production past caecum • Synbiotic and GBRS most adequately ↑ SCFA levels along the colon length
Chapter 6 – Efficacy of sugar cane fibre and probiotic spore synbiotic combination in attenuating chronic colonic inflammation in spontaneous colitic Winnie mice	

Disease parameter	Key findings
DAI, % body weight change and macroscopic markers	• Synbiotic and *B. coagulans* most effective in reducing diarrheic and bloody faeces (-69%), followed by GBRS (-60%) • *B. coagulans* (74%) followed by synbiotic (50%) and PSCF (33%) supplementations effective in preventing body weight loss • Synbiotic most significant in improving all tested macroscopic markers except for spleen weight • Only synbiotic prevented colon length shortening
Histology	• Synbiotic significantly ↓ histology score in both PC and DC sections • *B. coagulans* followed by PSCF ↓ histology score only in DC with no effect in PC
Immunomodulatory effects on colonic cytokine levels	• Synbiotic exerted consistently significant immunoregulatory effect in DC section with no effect on specific cytokines in PC • *B. coagulans* effective in regulating the cytokine levels differently in PC and DC, while no effect on certain cytokines (IL-10, IL-17) in either colonic sections • PSCF induced varying effects in PC and DC sections for certain cytokines and showed no effect for IL-6, IL-10, IL-12 and IL-17 in neither PC nor DC sections
Immunomodulatory effects on serum cytokine and CRP levels	• Synbiotic significantly induced modulations in only specific cytokine levels in serum while had no effect on the levels of IL-1α, IL-17, GM-CSF and MIP-1α • PSCF ↓ levels of limited cytokines in serum – IL-β, IL-6, MIP-1β and TNF-α • *B. coagulans* ↓ the levels of limited cytokines in serum – IL-β, IL-6, IFN-γ, MIP-1α and TNF-α • Synbiotic and *B. coagulans* more effective than PSCF in suppressing serum CRP levels
Microbiota modulations in caecal, mucosal-associated and faecal samples	• Synbiotic ↑ Shannon and Simpson indices in caecal and faecal samples • All Winnie samples showed clear demarcation from WT samples in terms of beta diversity • Synbiotic induced modulations in the levels of Firmicutes and Bacteroidetes and favoured abundance of Prevotella, while PSCF favoured abundance of Verrucomicrobia
SCFA profile of caecal, mucosal-associated and faecal samples	• Winnie-control mice showed significant alteration the SCFA levels relative to WT • Highest concentration of SCFA observed in caecum • *B. coagulans* lacked ability of eliciting SCFA production past caecum • Synbiotic and PSCF most adequately ↑ SCFA levels along the colon length

Different prebiotic fibres, due to their varying biochemical make-up, have been demonstrated to impart different effects on the host (75, 616, 617). The PSCF and GBRS exerted some unique immune-regulating effects, particularly in the distal colon. PSCF alone (Chapter 4) caused significant reduction in secretion of IL-1α and TNF-α with no effect on IFN-γ levels compared to the DSS-control. In contrast, GBRS supplementation (Chapter 5) had no effect on IL-α and TNF-α while, substantially suppressing IFN-γ levels. Moreover,

relative to DSS-control, PSCF caused more reduction in the secretion levels of colonic IL-1β (-64%) and IL-6 (-81%) compared to that induced by GBRS (-36% and -35% respectively). This is in agreement with a recent study (616) that showed that different prebiotic fibres modulate the immune response differently.

The two prebiotic DF components alone, also varied in term of their ability to enhance the SCFA production. GBRS separately, and in synbiotic combination (Chapter 5), was more effective in inducing elicited SCFA levels along the colon length than that mediated by either PSCF or its synbiotic combination (Chapter 4). This indicates more rapid microbial metabolization of the RS from GBRS than the slowly-fermentable cellular material from PSCF. The amounts and ratios of SCFA produced, as well as the rate of production, varies with different fibres (59, 617, 618). Rapidly fermented fibres however, may lead to excessive gas production and bloating, so dose is an important consideration (619). The fermentation pattern of fibres is known to be influenced by factors including molecular weight, chain length and the structure of the fibre (59). The higher dose of GBRS (400 mg/day) compared to the PSCF (200 mg/day) administered in the two studies could also contribute to the difference in the SCFA levels produced.

The loss of the ability by *B. coagulans* alone to elicit greater SCFA levels (either directly by metabolising available chow fibre or indirectly by stimulation of SCFA-producing gut bacteria via cross-feeding) beyond the caecum could be improved by addition of prebiotic fibre (as synbiotic) to generate complimentary/synergistic SCFA induction as evidenced in Chapter 4 and 5. While the *B. coagulans* spores were noted in the study to augment the SCFA production when applied as synbiotic, it was not confirmed whether this marked effect on SCFA levels was the result of either direct fermentation of fibres by *B. coagulans* vegetative cells or via in-direct means by influencing other SCFA producers in the gut. This could be more thoroughly studied by employing an *in-vitro* human digestive and gut microbiota model system using human faecal samples (75) to understand the changes induced by *B. coagulans* supplementation. Additionally, the *in-vivo* screening trials could also be conducted to understand the interaction of either vegetative cells or heat-killed spores with these prebiotic fibres. Such information will further help delineate whether the live spores, spore components (in case of heat-killed spores) or the vegetative cells are involved in inducing the beneficial probiotic effects.

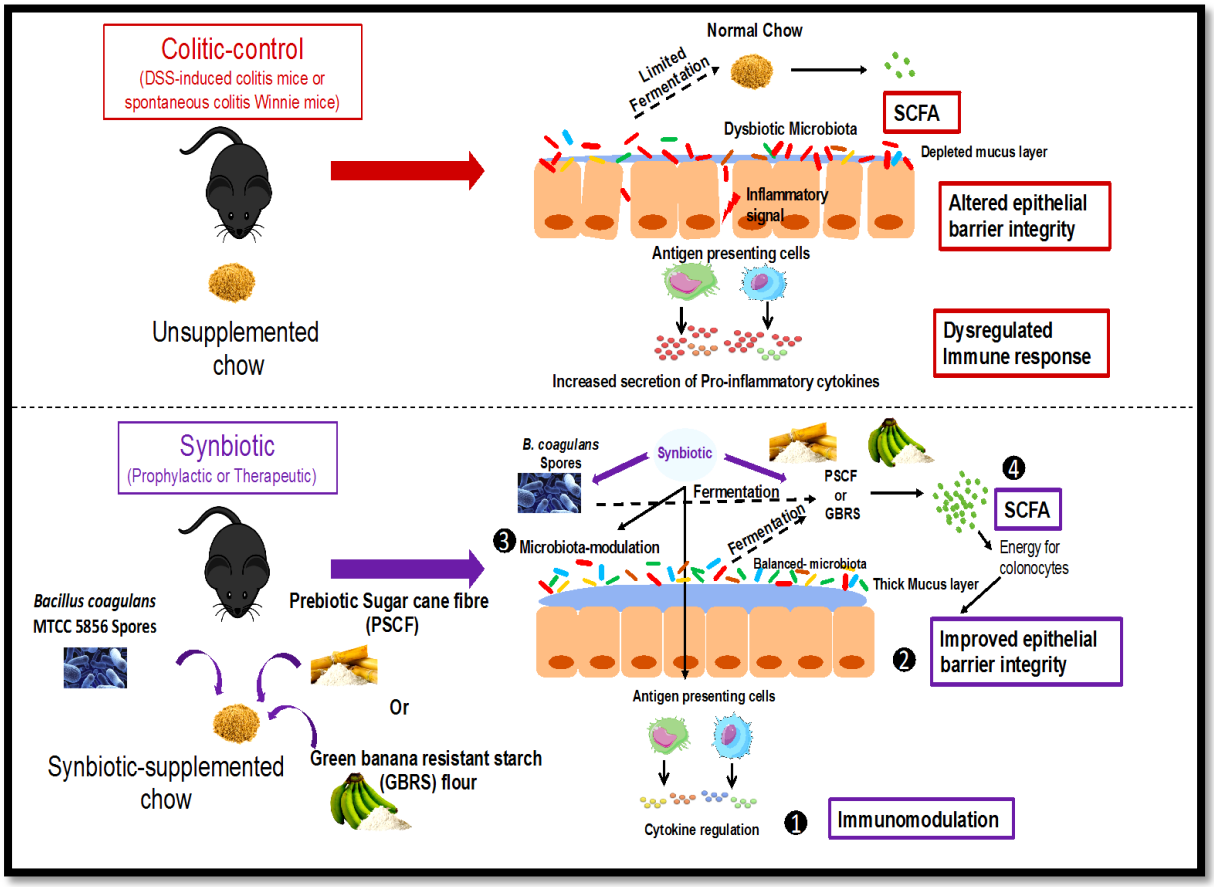


Figure 7.1. The potential mechanism of synergistic synbiotic application (prophylactic or therapeutic) targeting different inflammatory circuit components of IBD in mice models of colitis. The synbiotic health outcome effect noted may be associated with a synergistic direct immune-regulating efficacy of the probiotic and prebiotic components (1), their ability to protect epithelial integrity (2), stimulation of probiotic spores and/or native gut microbiota by prebiotic fibre (3), and/or with stimulation of greater levels of fermentation of fibres releasing SCFAs (4) that mediate reduction in colonic inflammation.

The animal model studies (Chapters 4, 5 and 6) in this research supported the application of the synbiotic combination as a two-point approach targeted at achieving augmented beneficial outcomes by encompassing the synergism between probiotic and prebiotic components. The potentiated effects of synbiotic combination observed in this research could be associated with its efficacy being due to multiple mechanisms of synergistic functioning that hinder the inflammatory circuit in IBD as illustrated in Figure 7.1. The study designs of the *in-vivo* mice feeding trials allowed determination of individual, as well as synbiotic outcomes, on the overall inflammatory status. The sacrifice of the animals allowed collection of serum and colonic tissues for cytokine measurements and histological grading as well as collection of contents from caecum and colon (mucosal) for SCFA and microbial profiling. These types of samples are often difficult to obtain in human studies, but the resulting molecular, biochemical and histological analyses of the colonic tissues helped determined the efficacy of the supplementations on the disease outcomes. This complete

SCFA and microbial profiling from the site-specific samples (caecal, mucosal-associated and faecal contents) was instrumental in delineating the synbiotic functioning. The synergistic synbiotic effect was more prominently noted as benefits that occurred along the colon length compared to the site-specific effects obtained using individual probiotic and prebiotic supplementation.

However, while the mice models did allow the modulations in immune parameters, epithelial integrity, microbiota and associated metabolites (SCFA) to be studied in the controlled experiment, direct human trials will be required (620, 621). The variations in the evolved biology of humans, compared to mice, demands caution in translation of the results to impacts on human health and diseases (219). In addition, a small sample size for molecular analyses is also acknowledged to be a limitation in direct translation of such effects in humans. Also, care should be taken to translate the amount fed as a supplement to mice to human dose and the organoleptic tolerability of the supplements being tested for human application.

7.2 Future research directions

To achieve the goal of delivering the synbiotic functional ingredients in influencing health and eating practices in humans, development of convenient on-the-go food delivery forms is projected. The heat-resistant capacity of *B. coagulans* spores and its synergy with the prebiotic fibres – PSCF and GBRS could be particularly taken advantage of to develop shelf-stable synbiotic food products. In this context, a snack bar is an ideal food matrix owing to its portability and as it allows storage without the need for refrigeration. A supplement snack bar carrying green banana flour is being currently evaluated for sensory acceptability among defence personnel in Australia (622). Such a food matrix could be further enhanced by adding probiotic ingredients as well as additional prebiotic fibres to boost the gut immunity, while allowing slow energy release and promoting satiety. The studies focussing on developing and testing the stability of the snack bar carrying functional synbiotic ingredients will be required to guide the refinement of the formulation. The neutral taste and texture of PSCF and the ability of GBRS to stabilise emulsions could be expected to improve textural, functional and nutritional profile of these nutrient-dense food products. Sensory testing to evaluate the organoleptic acceptance by the consumers will be needed prior to health efficacy testing. Such value-added synbiotic products will offer greater choices of improved dietary quality food products thus, influencing improved eating practices for optimal wellness.

A pilot human clinical trial in healthy volunteers (e.g. *n* = 30-40, 1-2 months duration) targeted for proactive prevention could be designed to determine if the consumption of synbiotic snack bar can reflect the beneficial changes that are seen with a healthy fruit and vegetable diet. The markers for health parameters could include testing for faecal microbiota and metabolomic profiling, serum CRP and serum/faecal cytokine profile (409). Besides providing the information on the efficacy of the tested synbiotic snack bar on health, such pilot trial could also allow determination of the dose responses and tolerability of ingredients in humans. The anti-inflammatory ability of synergistic synbiotic combinations tested in the research could also be applied to investigate its benefits in IBD patients through food delivery forms. However, any such research on synergistic combinations as a medical intervention would need to be conservatively applied in association with patients' existing drug therapy. The functional synbiotic snack bar carrying the potent identified bioactive ingredients also offers potential for evaluating its benefits for weight maintenance and chronic low-grade inflammatory metabolic disorders. Thus, the outcomes of this research could be applied to facilitate the paradigm shift from passive healthcare recipients to active health care consumers seeking optimal wellness through improved functional food choices to negate the effects of the westernised diet.

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Appendices

Appendix I. Nutritional information of Kfibre™ Prebiotic sugar cane fibre

Nutritional Information

Serving per package: 66 serving size: 1.5g (1 teaspoon).

A 100g bag is a 1 month supply for 1 person taking 2 serves per day.

Ingredients: 100% Sugarcane (sucrose reduced).

Kfibre™ contains NO artificial colours, flavours, preservatives or chemical stimulants.

Nutritional Panel

	Average quantity per serving	Average quantity per 100g
Energy	14kJ	915kJ
Protein	<0.1g	2.2g
Fat, Total	<0.1g	2.6g
-saturated	<0.1g	1g
Carbohydrate	1.4g	91.7g
-sugars	<0.1g	4.6g
Dietary Fibre	1.3g	87.1g
Sodium	<0.1g	23mg

Kfibre™ has no known allergens and each serve contains over 5% RDI dietary fibre.

Kfibre is produced with a chemical free production process. Due to this unique process, Kfibre retains all the goodness associated with a whole-plant dietary fibre.

Downloaded from https://www.kfibre.com/

Appendix II. Nutritional information of Natural Evolution™ Green banana resistant starch flour

Nutrition Facts

4.5 servings per pack
Serving size 3/4 cup (100g)

Amount per serving
Calories 338

	% Daily Value*
Total Fat <1g	**0%**
Saturated Fat <1g	**0%**
Trans Fat <1g	**0%**
Cholesterol 0mg	**0%**
Sodium <1g	**0%**
Total Carbohydrate 75g	**25%**
Dietary Fibre 12g	**43%**
Total Sugars 1g	
Includes 0g Added Sugars	**0%**
Protein 3.5g	**7%**
Vitamin D 0g	0%
Calcium 18mg	1.8%
Iron 0.94mg	5.2%
Potassium 1200mg	34%

* Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:

NUTRITION INFORMATION PER SERVE:
1 Serve = 100 grams

	QUANTITY/ 100g SERVE	%DAILY INTAKE*
Energy	1415kj	16%
Protein	3.5g	7%
Total Fat	<1g	0%
saturated	<1g	0%
monounsaturated	<1g	0%
Polyunsaturated	<1g	0%
Trans	<1g	0%
Dietary fibre	12g	40%
Carbohydrates	75g	24%
Total Sugar (sucrose)	<1g	0%
Sodium	<5g <0.2mmol	0%
Potassium	1200mg	40%
Zinc	5.5mg	8%
Magnesium	121mg	42%
Vitamin E	1.77IU	17%
Resistant Starch	27g	

*% Daily Intakes are based on an average adult diet of 8700kj per day. Your daily intakes may be higher or lower depending on your energy needs.

< means less than

Ingredients:
Green Bananas grown in Tropical North Queensland, Australia.

Made in Australia by Natural Evolution
corner Hansen & O'Connell Road, Walkamin, Queensland AUSTRALIA 4872

Appendix III. Nutritional information of mice standard chow diet

RIDLEY AGRIPRODUCTS

BARASTOC

Mice Cubes Irradiated 10kg

Key features:

- A complete and balanced diet to support growth and health of mice and rats in a laboratory environment.
- Product is Gamma Irradiated at a rate of 25 kilo gray.
- Pelleted product 12mm in diameter and an average of 20mm in length.

Presentation:

- 10kg net weight
- Packaged in a double walled paper bag, then placed in a 200 micron plastic bag, which is vacuum packed and heat-sealed. This sealed package is then sewn in a single walled paper bag and then packed in a cardboard outer carton for shipping.
- Product is packed 48 per pallet.

Ingredients:

- Wheat, Wheat Byproducts, Groats (Dehulled Oats), Meat Meal, Canola Oil, Soyabean Meal, Skim Milk Powder, Molasses, Salt, Vitamins, Trace Minerals.

TYPICAL ANALYSIS			
Min Crude Protein	20%	Vitamin D3	2 IU/g
Min Crude Fat	6%	Vitamin E	260mg/kg
Crude Fibre	3.2%	Vitamin K3	55mg/kg
ADF	4.2%	Vitamin B1	64mg/kg
NDF	11.2%	Vitamin B2	48mg/kg
DE_HORSE	12.8 MJ/kg	Vitamin B6	30mg/kg
Calcium (Ca)	1.14%	Vitamin B12	0.08mg/kg
Phosphorous (P)	0.94%	Niacin	400mg/kg
Sodium	0.35%	Panto	220mg/kg
Potassium	0.82%	Biotin	1.48mg/kg
Chloride	0.58%	Folic	11mg/kg
Magnesium	0.24%	Iron	51mg/kg
Lysine	1.11%	Zinc	60mg/kg
Methionine	0.37%	Manganese	120mg/kg
Linoleic	1.52%	Copper	10mg/kg
Starch	29%	Selenium	0.1mg/kg
		Molybdenum	0.4mg/kg
Added Vitamins and Trace Minerals		Cobalt	0.6mg/kg
Vitamin A	15 IU/g	Iodine	1.4mg/kg

Product code **102108**

For more information please contact Ridley Sales & Support on 1300 666 657
or visit www.agriproducts.com.au

BARASTOC COBBER Rumevite

Appendix IV. Copy of ethics approval permit

UNIVERSITY of TASMANIA

Animal Ethics Committee
ETHICS APPROVAL PERMIT

Office of Research Services
Phone : 03 62267283
Fax: 03 62267148
animal.ethics@utas.edu.au

To: Dr Raj Eri

From: Marilyn Pugsley

Date: 09 August 2016

Project: A0015840 - Efficacy of prebiotic and probiotic combinations in a food delivery format for improved gut health

Approved on: 09 August 2016

Approval expires: 09 August 2019

1st Annual Report due: 09 August 2017

Please read this permit carefully as **approval may be withdrawn** for non-compliance with the conditions stated below.

The Animal Ethics Committee has approved the above project and a copy of the initial application document is attached. The approval is subject to the review and AEC approval of an annual report which is due before the approval anniversary. **Please note the due date in your diary.**

As the Responsible Investigator, you MUST ensure that:

1. All aspects of the work conform to the requirements of the current edition of the *Australian code of practice for the care and use of animals for scientific purposes* 8th edition 2013
2. The project is conducted in accordance with the provisions of the Tasmanian Veterinary Surgeons Act 1987 and Veterinary Surgeons Regulations 2012. If the project involves a veterinary service or other animal service, it is **your responsibility** to contact the University Veterinarian to discuss the legal requirements of competency assessment.
3. The University Veterinarian and the Animal Ethics Committee are promptly notified of any unexpected event which was not considered in the initial application and impacts on the welfare of any animal directly or indirectly involved in the project.
4. You contact the University Veterinarian to advise when and where your experiments will be conducted. Sufficient notice needs to be given so that an inspection can be easily arranged.
5. In the event of any unexpected death, you contact the University Veterinarian to perform an autopsy.
6. A full record is maintained of all animals used in this project. If at any stage you anticipate the need to use additional animals this must be communicated to the committee before use. Using additional animals without AEC approval is a breach of your ethics permit.
7. That all investigators attend Ethics training sessions every three years. Contact the Executive Officer Animal Ethics for the next available session.

The project is approved for a maximum of 3 years. If the project is to continue past the expiry date, a new initial application will need to be submitted.

If the investigation necessitates a Parks & Wildlife permit or other permits, you are required to send copies to animal.ethics@utas.edu.au before commencing work.

Executive Officer
Animal Ethics Committee